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Contract No. W-7405-eng-26

PROCEEDINGS OF THE WORKSHOP ON SUBCHRONIC TOXICITY TESTING

Denver, Colorado
May 20-24, 1979

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Work sponsored by the Office of Pesticides and Toxic Substances,
U.S. Environmental Protection Agency, Washington, D.C., under
Interagency Agreement No. 80-D-X0453

Date Published: November 1980

OAK RIDGE NATIONAL LABORATORY
OAK RIDGE, TENNESSEE 37830
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UNION CARBIDE CORPORATION
for the
DEPARTMENT OF ENERGY

At the time of publication, we were saddened to learn of the death of a workshop contributor, Bernard McNamara. His scientific and personal advice will be missed by those who worked with him on this and other projects.

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PREFACE

This document is the product of a three-day workshop sponsored by the Office of Pesticides and Toxic Substances of the Environmental Protection Agency (EPA) to evaluate the subchronic toxicity test and its role in chemical assessments mandated under the Toxic Substances Control Act (TSCA). The Health and Environmental Studies Program of the Oak Ridge National Laboratory (ORNL) had the responsibility for the coordination of the workshop and the preparation of the resulting proceedings. The participants, selected to achieve a balance of industrial, academic, and governmental viewpoints, were provided with a review of the pertinent literature prepared by ORNL. Because many of the topics scheduled for discussion had limited support in available literature, much of the document represents the results of the committees' discussions, analyses, and recommendations.

The workshop was divided into five committees, each with a specific area for discussion. The proceedings of the workshop are a consolidation of the five committee drafts into one report, thereby enhancing the cohesiveness of the rationale and avoiding repetition in the discussions.

In preparing this report, both during and after the workshop, several individuals were especially helpful. Dr. Witschi of the ORNL Biology Division advised the ORNL staff on technical matters and was responsible, in large part, for the initial organization of the workshop and steering committee. The steering committee members, Drs. Galbraith, Gibson, Oehme, Peck, Pfitzer, Page, Sawhney, and Terhaar, contributed much time and effort to the refinement and completion of this project. The success of the enterprise was also due to the guidance provided by the EPA Deputy Assistant Administrator, Dr. Muir, and the EPA Senior Medical Advisor, Dr. Seifter. Foremost among the reasons for the success of the workshop, however, was the enthusiastic and altruistic attitudes of the participants, who, despite differences in scientific opinion, maintained friendly, open-minded discussions.

Because the EPA desired a general evaluation of subchronic toxicity testing, this workshop addressed the major issues. No attempt was made to cover every aspect of assessment. That some issues are not addressed in the report does not mean they are less significant. The committees identified areas in which research is needed and problems of test design unresolved during the workshop. Recommendations based on these findings were an important product of the committee discussions.

KEYNOTE ADDRESS TO THE WORKSHOP PARTICIPANTS

WARREN R. MUIR
U.S. Environmental Protection Agency

I would like to welcome all of you here to Denver and thank you for participating in this workshop. It is an undertaking that we at the Office of Toxic Substances and the Environmental Protection Agency (EPA) feel to be very important. We certainly appreciate that you are attending and helping us with this effort.

I am Warren Muir and I head the Office of Testing and Evaluation, which is one of the three offices set up to implement the Toxic Substances Control Act (TSCA) in EPA. As most of you are aware, TSCA was passed in 1976 as a particularly important new piece of legislation dealing with chemical substances and their hazards. Unlike the 15 or so preceding acts, which also address regulation of chemical substances, TSCA is unique in its authorities to generate health, environmental, and other chemical information from the industry which manufactures or proposes to manufacture chemicals. Therefore, in our efforts to support our direct regulatory controls and to increase our understanding of the nature of the chemicals before they enter our environment, we are especially emphasizing the TSCA aspects of reporting, record keeping, information systems, testing, and evaluation of information.

The Office of Testing and Evaluation, the scientific office of the three implementing offices, is responsible for the definition of testing in terms of both requirements and guidelines. The Office is also responsible for compiling information available to the EPA (including information to be generated under this testing), analyzing it, and evaluating it to formulate risk assessments on chemicals.

With respect to testing, there are two provisions that are applicable and in which we are heavily involved. One is Section 4 of TSCA, a section of the act which allows EPA to specify both test standards and chemicals to which the test standards would apply. It basically requires either processors or manufacturers or both to conduct testing on high priority chemicals. These are chemicals which the EPA may define as posing an unreasonable risk or which are produced in substantial quantities with either substantial releases to the environment or substantial risk for human exposure.

With respect to Section 4, the first set of test standards, which include oncogenicity, chronic toxicity, and the combined oncogenicity and chronic toxicity protocols, were published in proposed form in the *Federal Register* about two weeks ago [9 May 1979]. Also published were the Good Laboratory Practices that were patterned after the proposed Food and Drug Administration's Good Laboratory

Practices for nonclinical health effects studies. With respect to the topic for this meeting, subchronic toxicity testing, we intend to propose, in a couple of months, subchronic toxicity test protocols along with acute toxicity, mutagenicity, reproduction, and teratology tests. The proposal, which will be published in the *Federal Register*, will be a reproposal of the test protocols that have been previously proposed by the Office of Pesticides Programs under Section 3 of their pesticide guidelines for the Federal Insecticide, Fungicide, and Rodenticide Act. We are proposing those standards in an effort to focus upon the various issues associated with those test protocols and to allow us the time to get together with the Office of Pesticides so that in going final with our test standards and test guidelines, that office and ours can merge efforts to come up with protocols that are identical to the maximum extent allowable under the two different laws. In addition, we are also working with the Interagency Regulatory Liaison Group, to make sure that there is maximum consistency not only across EPA offices in defining acceptable testing methodologies, but also across the federal regulatory agencies.

The other section of TSCA in which testing comes into play is Section 5, which applies to new chemical substances, new chemical substances being those that are not contained on the TSCA chemical inventory of all existing chemicals in commercial use. The inventory has been published. It is being distributed now, and *Federal Register* notices are going out indicating the official date of the inventory publication to be June 1. On July 1, thirty days after publication, the premanufacturing notification review process will be initiated.

The premanufacture notification review (mandated under Section 5) will consist of evaluating certain information about the chemical's identity, use, production, exposure, etc., and also all available health and safety studies on the material that is being proposed for manufacture. We do not generally have the authority under Section 5 to require testing. However, testing of new chemicals can be required if they fall under Section 4, in particular, if they fall in categories of chemicals that might be specified under Section 4. Also, under Section 5(e), the EPA has the authority to issue an order to limit production, distribution, or use as necessary if there is insufficient information provided to the EPA to enable it to determine whether the chemical poses an unreasonable risk, or if the EPA can demonstrate that the chemical may pose an unreasonable risk, or if the chemical is produced in substantial quantities with substantial release or exposure.

Because of the Section 5(e) provision in which the EPA may limit exposure or production in some fashion and because of concerns by industry and others as to what the EPA expects and will be satisfied with, we have contemplated coming forth with Section 5 testing guidance, which would describe the type of testing and the testing scheme or approach we would most like to see performed. To date, this has been a very controversial area, as I am sure many of you are well aware. We have had a number of open public meetings and have decided to move along fairly deliberately in this area before we propose the document. As a result we decided that rather than publish a formal proposal, we would first publish a discussion document which laid out the various alternative approaches available to the EPA for testing and then suggest a menu of tests we thought might be appropriate to incorporate into a future testing guidance. Also, we want to solicit as much public comment as possible regarding both

the scientific merits of the tests and the various arguments, scientific and other, which might impinge upon the choice the EPA would make among the various available alternative testing approaches. That discussion document was published March 16 in the *Federal Register*. It is still open for comment, and we are getting a large response.

With respect to assessments, our office is involved in four different types. One is under the chemical substances premanufacture notification process. Another is a review of chemicals to see if they meet the Section 4 criteria for testing, and two others deal with existing chemicals. These last two include our comprehensive assessment process and a priority problem assessment, the priority problem assessment being a subset of comprehensive assessment and a faster track. These latter two are multistaged assessments of existing chemical substances, multistaged primarily so that we can take the limited amount of resources available in our program and target them as efficiently as possible on the highest priority problem chemicals. This focuses our limited energies on those chemicals of greatest importance. Therefore, we do basically a quick and dirty analysis on a very large number of chemicals and then narrow down to what in the last analysis are many fewer chemicals.

In the later stages of chemical assessment, we go through all the relevant scientific information in an effort to pull together as good a risk evaluation as possible and use this evaluation to base actions or potential actions under TSCA or other regulatory statutes. Because of both our testing authorities and our assessment activities, we are particularly interested in evaluating the state of the art of various testing approaches, in particular today, to focus upon subchronic toxicity testing. We are very interested in the state of the art of this type of testing method in the context of TSCA authorities and of our ability to use it in terms of both our testing schemes and our evaluations of information.

We are particularly interested in this workshop and addressing questions such as proper protocol designs and the extent to which protocol design should be very specific or more general. We recognize that under Section 4 of TSCA, the EPA has to specify beforehand what testing should be done and is not able to turn away inadequate studies later, as long as the studies meet the required specifications. In this regard, the EPA is unlike the Food and Drug Administration and the Office of Pesticides Programs, which register materials only after acceptable studies are performed.

Further, we are interested in any judgments the workshop can make with respect to the relevance of the subchronic toxicity test results for risk assessments. We are also interested in the judgments of this particular group with respect to the relationship of subchronic toxicity tests to other types of tests, such as acute tests or chronic tests in an evaluation scheme. For example, Dr. Seifter, my Medical Science Advisor, is of the opinion that perhaps a subchronic toxicity test does not have a place in a TSCA evaluation scheme at all, that we may be able to substitute a shorter test of 14 or 28 d with considerably more information on metabolism, biochemistry, etc., and then, depending upon the results, follow directly with a chronic test. We would be interested in the views of this group on this idea and others.

I presume that there will be ample opportunity to ventilate that particular proposal. We are, therefore, interested in these types of activities, and you can understand why this particular workshop is so important to our office. Because of our

interest we asked the Oak Ridge National Laboratory to assist us in establishing this workshop, and we are very pleased with the group we have been able to assemble here to meet this week. We deeply appreciate the sacrifices each and everyone of you has made in your busy schedule to come out here, roll up your sleeves, and work with us on this agenda. Thank you.

REVIEW OF CURRENT ACTIVITIES IN TEST STANDARDS DEVELOPMENT

NORBERT P. PAGE
U.S. Environmental Protection Agency

During the last few years, increased legislative responsibilities have been assigned to government agencies in both the U.S. and abroad to better evaluate environmental chemicals for their potential toxic hazards. Safety assessment has been routine in the pharmaceutical industry for years. Safety testing of food additives and pesticides and, to a lesser extent, industrial chemicals has also been conducted. Now virtually all chemical manufacturers are responsible for developing data to demonstrate the toxic potential of their products, regardless of their products' intended uses.

Many laws have been passed which require that the government agencies develop standards or guidelines by which testing must be conducted. With these new demands, a flurry of activity has taken place both in the U.S. and abroad in the test standards or guidelines area. Dr. Muir has already spoken to the specific needs of the EPA in fulfilling its mandates under the Toxic Substances Control Act.

It is my intent in this presentation to demonstrate how the EPA relates to other ongoing activities in the testing area. Four different acts within the purview of the EPA alone require the testing of chemicals and the development of testing guidelines. These are the Toxic Substances Control Act (TSCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Clean Air Act as amended in 1977 (fuels and fuel additives), and the Resources Conservation and Recovery Act (hazardous wastes).

Among the other regulatory agencies, the Consumer Product Safety Commission (CPSC), through the National Academy of Sciences (NAS), revised its procedures for toxicity testing of household products (NAS 1977). This was completed about two years ago. The Food and Drug Administration (FDA) is now engaged in a cyclic review of approximately 2100 direct food additives. They are developing procedures for toxicity assessment including identification of test procedures. The Food Safety Council (1978) has also produced a series of test procedures. In an FDA-related activity, the Pharmaceutical Manufacturing Association has drafted its Preclinical Guidelines for Assessment of Drug and Medical Device Safety in animals.

On the international scene, several foreign countries, the World Health Organization (WHO), the European Economic Commission (EEC), and the Organization for Economic Cooperation and Development (OECD) have been producing test guidelines. I am sure I left out a few other ongoing efforts. Many of you have participated in these guideline activities.

One could pick from any number of protocols, and having such a wide range of choices may at first seem to be an advantage. On the other hand, conflicting methods may be required of the manufacturer of a product—especially where there is more than one use—to satisfy the requirements of several agencies or countries.

Attempts at Harmonization

What is being done to harmonize guidelines? Harmonize—that is the new word about town that sounds better than, but means about the same as, standardization.

Within the EPA, the Office of Pesticides Programs and the Office of Toxic Substances have joined together to develop a common set of guidelines. Those implementing the Clean Air Act in the testing of fuels and fuel additives are committed to using the TSCA/FIFRA guidelines rather than producing their own. Thus, the EPA will basically have a single set of toxicity testing guidelines.

Within the federal bureaucracy, the regulatory agencies have banded together to come up with consistent and cooperative regulatory actions. To achieve this, the Interagency Regulatory Liaison Group (IRLG) was formed. One work group was chartered specifically for toxicity guidelines and standards. Five federal regulatory agencies participate: EPA, FDA, CPSC, the Occupational Safety and Health Administration, and the U.S. Department of Agriculture. Dr. Victor Morgenroth (FDA), the chairman, is participating in this conference. When IRLG develops final guidelines, the agencies will adopt them.

As far as the International circuit is concerned, the U.S. is an active participant in both the WHO and OECD programs. Of the two, the OECD will likely carry the most clout. Participating countries will likely accept the methods developed and incorporate them into their chemical control laws.

Status of Activities/Concept of Progress

Last August under FIFRA, the EPA proposed pesticide registration guidelines for the major health effects. On May 9, the EPA proposed chronic standards and Good Laboratory Practices (GLPs) under TSCA. It is expected that in July or August most of the remaining TSCA standards for health effects testing will be proposed. These will be essentially the same as the FIFRA guidelines. By the end of the year or in early 1980, joint TSCA/FIFRA guidelines should be ready for finalization in the Code of Federal Regulations. The IRLG is also participating with the EPA in reviewing public comments and revising the proposed guidelines.

The IRLG will soon propose a series of guidelines for acute effects and teratogenicity. It is expected that subchronic guidelines will be ready for agency reviews by late summer and those for chronic tests, by fall.

The OECD has formed several groups of experts to prepare universally acceptable guidelines. The U.S. has the lead in establishing guidelines for chronic effects and GLPs, the British, for short-term effects, and Sweden, for step-systems. Other groups are also working in ecotoxicity, biodegradation, bioaccumulation, etc. Proposed guidelines are now prepared for eye and skin irritation, acute and subacute toxicity, and teratology. As with the IRLG, it will be summer before the OECD has drafted a subchronic test protocol.

Review of Recently Proposed Subchronic Toxicity Tests

Table 1 lists the basic experimental design for four of the recent activities that I mentioned. There are no great surprises here, as the designs are similar to those that have been in use for several years. They all call for a fairly detailed clinical and pathological examination, including microscopic examinations. There are some differences in organs to be weighed, tissues to be examined, etc. These differences should be reviewed by the appropriate work groups. Whereas there is fair consistency in certain design aspects, other areas are controversial (e.g., duration, species). The FDA's draft for food additives testing is very similar to that for the IRLG shown in Table 1.

In addition to the 90-d oral subchronic tests, other subacute or subchronic tests have recently been proposed. The FIFRA guidelines provide a separate protocol for a 90-d subchronic inhalation toxicity test. The basic design is similar to that presented for the 90-d oral test, except that only the rat is required, and the number per group is only 10 instead of 20. The remainder of the design is virtually the same but with increased clinical and pathology examination of the respiratory system. The IRLG has not yet prepared guidelines for a subchronic test by the inhalation route.

The proposed FIFRA guidelines and the IRLG guidelines also include dermal toxicity tests of 90-d durations. In both, albino rabbits are the species of choice. Eight to ten young adults of both sexes are used for each of three dose levels plus controls. The highest dose is to be selected to produce toxic effects but not cause excessive mortality (over 10%). The lowest dose should be a no-observed-effect level.

The FIFRA proposed guidelines provide a 21-d dermal toxicity test for products whose pesticidal use is likely to result in repeated human skin contact. This is not needed when a 90-d dermal toxicity test is performed. Except for duration, the protocol is the same as for the 90-d dermal test.

The FIFRA proposed guidelines require a delayed neurotoxicity test with mammals if neurotoxicity was observed with acute tests. The species to be tested is that which demonstrated the neurotoxicity signs. Both sexes are to be used for mammals, whereas only the female domestic chicken is used. At least three dose levels each with 10 animals of each sex are employed with exposure for a 90-d period. The highest dose is one that induces toxic effects, whereas the lowest should not induce any deleterious effect. Observations for behavioral abnormalities, including locomotor activity, are called for, along with very detailed and complete pathological examination of the central nervous system and peripheral nerves.

Table 1. Experimental design for recently proposed subchronic oral toxicity tests[a]

	FIFRA guidelines (1978/1979)[b]	Interagency Regulatory Liaison Group (1979)[c]	World Health Organization (1978)	Food Safety Council (1978)
Species	Two (rat and non-rodent)	Two (rat and dog)	Two (rodent and non rodent)	Two (rodent and non rodent)
Sex	Both	Both	Both	Both
Age at start	Rats—6 weeks Dogs—4 to 6 months	Rats—6 weeks Dogs—4 to 6 months	Rats—just after weaning	Not indicated
No. of animals per group (sex/dose)	Rats—20 Dogs—6	Rats—20 (30 d to 6 months) 10 (< 30 d) Dogs—4	10	Rodents—10 Nonrodents—3 to 4
Duration	Rodents—90 d Dogs—6 months	Generally 90 d; shorter for special situations	10% of lifespan or 3 months	Rodents—90 d to 1 year Nonrodents—1 year
No. of doses	3	3	4	5
Dose levels	Highest—effect level but not more than 10% mortality Lowest—no evidence of toxicity	Highest—toxic level but not excessive mortality Lowest—no evidence of toxicity	Highest—distinct toxic level Lowest—no detectable toxicity reaction	Highest—clearly toxic but not lethal Lowest—no observable adverse effect but a reasonably large multiple of estimated human daily intake

[a] Minimal requirements.
[b] Proposed or unofficial guidelines under the Federal Insecticide, Fungicide, and Rodenticide Act. Control groups are required.
[c] Proposed or unofficial guidelines. Control groups are required.

The 28-d Toxicity Test

The FDA is proposing a 28-d toxicity test that they refer to as a range-finding oral toxicity study. It is intended to determine the target organs and find a range of doses for subchronic or chronic tests. It is similar to the 90-d IRLG oral subchronic test, except for a 28-d exposure and fewer animals per group (i.e., 10 rats instead of 20 and 2 dogs instead of 4 or 6).

The OECD/EEC has also proposed a 28-d test using the rat only, but it is otherwise like the FDA test. The 28-d test is to be used in a step-sequence system. The concept of the step-sequence scheme is to determine the tests to be performed based upon the potential for exposure and effects. The current sequence for testing new chemicals as proposed by the EEC and OECD is as follows: for less than one ton, no tests are needed. For all chemicals produced in quantities of one ton or more, a series consisting of acute toxicity tests, a battery of two or three mutagenicity tests, and the 28-d test are required. For those chemicals to be produced in quantities greater than 10 tons, more testing is needed (e.g., fertility test, teratology tests in one species, more mutagenicity tests, and a 90-d subchronic test). At production of 1000 tons, the ante goes up even further in that chronic tests and pharmacokinetic studies would be undertaken.

I hope from this quick review you can see the role that the subchronic test will play in the U.S. and international plans for safety assessment of chemicals. The subchronic test, more than any other, plays a pivotal or key role in virtually all safety assessment schemes. Much of the driving force behind protocol design by OECD and TSCA for new chemicals is that of economics. We recognize that testing costs can stifle the introduction of new chemicals and that inconsistent testing requirements can create international trade barriers. These are both issues of concern to EPA in its implementation of TSCA. This workshop needs to examine the trade-offs in sensitivity and usefulness of the subchronic test if we reduce requirements (e.g., 2 species to one, 90 d to 28 or less, reducing clinical or pathology requirements, etc.). We need solid data to predict the consequences of such modifications in test design.

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CHARGE TO THE WORKSHOP AND THE INDIVIDUAL COMMITTEES

WAYNE M. GALBRAITH
U.S. Environmental Protection Agency

A subchronic toxicity study can be defined as qualitative and quantitative evaluation of the physiological and pathological changes in the rodent and/or nonrodent resulting from regularly repeated exposure to a chemical over a period of one to three months.

It is generally recognized that toxicity resulting from acute chemical exposure and that resulting from subchronic and/or chronic exposure may differ. The results of acute studies may not be adequate to predict toxicity which results from repeated low-dose chemical exposure. Therefore, the subchronic toxicity study is fundamental in determining the toxicity profile of chemical substances to which repeated or continuous human exposure can occur.

It is the objective of this workshop to summarize the various factors which should be incorporated into the experimental design of an "end result"[1] subchronic toxicity evaluation and to examine the scientific basis for these factors. The ultimate objective of the workshop is to provide the rationale for cost-effective and scientifically sound subchronic toxicity testing procedures.

The committees should summarize the historical data bases which justify the inclusion of the various parameters in a subchronic study. When it is determined that expert opinion cannot be substantiated by an adequate data base, research recommendations should be made. In the course of your deliberations, all committees are also requested to devote attention to defining the technical terminology used in the committee documents.

It is not the objective of this workshop to duplicate what has been accomplished in a number of reviews and proposed test systems published in recent years (National Academy of Sciences 1975, 1977; World Health Organization 1978; Food Safety Council 1978; U.S. Department of Health, Education, and Welfare 1971; Association of Food and Drug Officials of the United States 1959). Nevertheless, some duplications of previous efforts are unavoidable and desirable to the end that the workshop product is comprehensive. When appropriate, the committees are encouraged to summarize and update pertinent information in previous reviews.

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[1]The term "end result" is intended to distinguish a study designed as the terminal toxicologic evaluation of the toxic effects of a chemical from a subchronic study used to determine dose levels for a chronic study.

The workshop should focus on the testing of chemicals covered by the Federal Insecticide, Fungicide, and Rodenticide Act and the Toxic Substances Control Act. The workshop is divided into committees which will address five general topics pertinent to subchronic toxicity evaluation.

The first committee will discuss general experimental design. Considerations such as minimum test duration, dosage, number of animals, test species, and animal age at time of chemical exposure will be evaluated by this group. For example, many scientists have difficulty agreeing with the requirement of a maximum permitted mortality in the high dose group in a subchronic study. Is this requirement justified? Would it be preferable to employ 10–15 rats per sex/dose group and have 5 or 6 dose levels rather than 3 dose levels with 20 rats per sex/dose group? What are the advantages and disadvantages of each study design?

The second committee will review parameters evaluated in a subchronic toxicity study. This includes general observations, clinical laboratory tests, and pathologic evaluations. A suggested minimum base set of general observations for rodent and nonrodent subchronic toxicity studies should be developed. It is also requested that the second committee address the desired frequency of chemistry and hematologic evaluation.

It has been suggested to the Agency that total serum protein, serum albumin, and serum protein electrophoresis are a better monitor of liver toxicity than serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase. Is there an adequate data base substantiating this statement? Are cholesterol and uric acid determinations justified in rodent and nonrodent subchronic toxicity studies? Should sperm motility be evaluated in control and treatment groups at the time of sacrifice? Are there other tests not routinely performed which should be? Are there special techniques, histochemical or other, which can be employed to increase the sensitivity of a subchronic toxicity study for detecting neurotoxicity? What comprises an adequate neurologic examination in the rat and dog in a subchronic toxicity study?

It is recognized that no toxicity testing protocol will be adequate in every experiment. For this reason the third committee has been asked to evaluate the relationship of protocol design to chemical class and characteristics. What observations, clinical laboratory tests, and/or pathologic techniques not routinely made should be included in protocol on the basis of chemical class? That is, what modifications in protocol design should be made on the basis of the physical-chemical properties of the test substance? What are the hazards or pitfalls associated with the subchronic evaluation of specific chemical classes? Examples that readily come to mind are the need to evaluate methemoglobin when testing nitrates and nitrites, the need for a cholinesterase activity evaluation when testing an organic phosphate or carbamate pesticide, and the potential aspiration hazard when conducting an oral-dosing study with certain hydrocarbons.

The fourth committee will evaluate the criteria to be employed for extrapolations from one route (i.e., oral, dermal, inhalation) to another route of administration. Because the cost of a subchronic inhalation study is at least twice that of a comparable oral-dosing study, it is imperative to know when inhalation

studies should be performed and when studies employing other dosing routes will be adequate. In this regard, what role should the results of acute inhalation and oral-dosing studies play in determining the need for subchronic studies using more than one route of administration? What criteria will be used to extrapolate from oral-dosing studies to inhalation studies? What assumptions must be made? What other studies, if any, must complement oral subchronic studies in order to make an extrapolation?

The fifth committee will evaluate the limitations of subchronic toxicity studies. What subchronic findings should trigger the requirement for a chronic study? What manifestations of toxicity are not likely to be observed in a subchronic study but may be found in a chronic study? How much information regarding maximum tolerance levels for humans can be obtained from a subchronic study? Is comparative metabolism the key factor in evaluating a toxicity profile of the same chemical in the rodent and nonrodent? If this is the case, is it possible to eliminate the need for a complete study in one species when it is determined that the adsorption, distribution, metabolism, and elimination pattern is similar for the rodent and nonrodent? How useful are the results of acute toxicology studies for determining if a metabolic difference exists in the rodent and nonrodent?

Other potential topics for consideration by this committee are the desirability of performing combination tests (e.g., subchronic and teratology in the same study), dosage extrapolation from laboratory animals to man, and results of acute toxicity which should trigger subchronic studies.

By Thursday morning each committee should have prepared draft documents which address the assigned topics. The document may also contain research recommendations and discussions of additional problems which the participants consider pertinent to subchronic toxicity testing. Of course, because of time constraints, it is recognized that all of the suggested topics may not be discussed.

Following the workshop, each participant is requested to make additions, corrections, or other changes in his or her committee's draft document and forward them to the appropriate chairman.

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PROCEEDINGS OF THE WORKSHOP ON SUBCHRONIC TOXICITY TESTING

1. INTRODUCTION

1.1 Scope

Under the Toxic Substances Control Act (TSCA) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Environmental Protection Agency (EPA) is required to provide standards or guidelines for the testing of chemicals for health and environmental effects. The EPA is also required to perform hazard or risk assessments for selected existing chemicals in the environment as well as for all new chemicals to be manufactured in the United States or to be imported. In addition to EPA's legislative requirements, other U.S. federal regulatory agencies and other countries have similar requirements to provide testing guidelines or guidance. The EPA has joined with other U.S. agencies in forming the Interagency Regulatory Liaison Group (IRLG) and with other countries in the Organization of Economic Cooperation and Development (OECD). Both the IRLG and the OECD have work groups to design testing procedures for the development of data for risk assessment of chemicals.

The subchronic study is a key test in assessing the relative safety of chemicals to which repeated or continuous exposure to humans can occur. This test is likely to be a fundamental part of any guidelines for the toxicity testing of new chemicals entering the market.

1.2 General Principles

Subchronic toxicity procedures are designed to determine the adverse effects that may occur with repeated exposure over a part of the average life span of an experimental animal. The boundary between the subchronic and chronic dosing regimes is often taken as 10% of an animal's lifetime; thus subchronic will include dosing regimes described as subacute (Organization for Economic Cooperation and Development 1979). Subchronic studies often involve multiple dosage levels, some of which may exaggerate by significant multiples the anticipated levels of human exposure. Testing procedures normally include the routes of exposure expected for man. Usually one, two, or more animal species are exposed to the chemical for a period of 90 d to evaluate the potential hazard. In the United States, one rodent (the rat) and one nonrodent species (the dog) are generally employed as the two species. In some other countries, the mouse, rather than a nonrodent, is used as a second species. The selection of animal species for subchronic studies should consider,

whenever possible, whether the test species' metabolic handling of the chemical or its analogs is similar to that of humans. Exposure levels used in the studies are lower than those in the acute toxicity procedures, and lethality is usually not an end point. Instead, by including many types of observations (behavioral, growth rate, hematological, biochemical, and functional) and extensive pathological evaluations, more subtle types of adverse effects can be detected.

Man may be exposed for the majority of his lifetime to low levels of a wide variety of chemicals. Usually the degree of exposure is insufficient to produce overt signs of toxicity; thus cause-effect relationships cannot be easily established. Epidemiological studies may assist in this respect, but because man is often exposed simultaneously to several chemicals, it is difficult to establish unequivocally the degree of hazard associated with any one chemical.

1.3 Objectives

For purposes of this report, the subchronic toxicity study is defined as an "end result" study, that is, it will be the only long-term study available to the regulatory agency responsible for making decisions about the risks of human exposure to a given chemical. Therefore, the subchronic study should define target organs and tissues in a manner sufficient to provide the basis for risk estimation. The studies should provide (a) the demonstration, with appropriate methods, that the toxic effects observed at the high-dose level are not observed at least at the low-dose level and (b) the delineation of tests that would also evaluate potential effects from long-term exposure conditions. The results from the subchronic study should be applicable to potential exposures of longer duration; however, it is not expected to be all-encompassing and predictive of such effects as carcinogenicity, teratogenicity, certain reproductive effects, or effects on longevity.

The workshop objectives included

- critically examining the subchronic study as it might be applied to general chemical testing programs and evaluating the effect of variations in study parameters on assessment potential;
- identifying the deficiencies in the existing scientific knowledge and recommending research to strengthen the testing program; and
- identifying the related scientific issues to be resolved in future workshops or experimental programs.

2. RELATIONSHIP OF PROTOCOL DESIGN TO CHEMICAL CHARACTERISTICS

The experimental design is based on the utilization of information available from physical and chemical data, acute studies, and structure-activity analogies with known substances. Variations in subchronic toxicological evaluations may be indicated for different chemical classes and characteristics. However, current knowledge does not permit development of a specific and definitive test design for each identifiable chemical class.

The outline design is based on the fundamental premise that a subchronic study is rarely conducted in the absence of prior information on the test chemical. The physical and chemical data listed in Table 2 will usually be known for the test substance. In addition, the investigator will usually have an indication of the acute toxicological response. It is this information (Table 3) considered together with the physical and chemical properties and, when available, the biological activity of analogous structures that will determine the design of the subchronic study. Other experimentally derived data regarding biological disposition of the toxicant, if available, should be utilized.

Table 2. Chemical and physical information

Molecular weight
Molecular formula
Structural formula
Electrophile/neutrophile
Purity
Acidity/alkalinity
Particle characteristics[a]
Density[a]
Corrosivity
Solubility in H_2O
Lipid solubility
Melting point[a]
Boiling point[a]
Vapor pressure[a]
Dissociation constant (pK)
Stability at various pH's
Stability to heat and light

[a] Relative to route of administration.

Table 3. Acute toxicity information

Time to onset of signs as a function of dose
Time to recovery
Weight changes
Organ effects
Observation of skin, fur, eyes, mucous membranes, etc.
Changes in behavior
Signs of autonomic nervous system effect, such as tearing, salivation
Changes in respiratory rate; depth and signs of respiratory irritation
Cardiovascular changes, such as flushing
Central nervous system changes, such as tremors, convulsion, coma
Observation of dropping pans for evidence of eating or noneating, urine color and volume, diarrhea
Time of death
Necropsy findings
Slope of the dose response line

The information depicted in Fig. 1 allows the investigators to make one of the following choices: (1) adequate data are available to predict hazard and a subchronic test is not necessary; (2) adequate data are not available to predict hazard and a routine subchronic test is needed; (3) the data indicate that because certain effects are expected, a modified subchronic study utilizing an expanded protocol with nonroutine tests should be designed; and (4) the data indicate the need for a chronic test that must be designed through use of a modified subchronic test. In some cases additional information may be available to indicate that no more work is necessary. This information may include available experimental data and such factors as the use, distribution, and production volume of the compound, as well as the likelihood of human or environmental exposure.

Structure-activity correlations can sometimes provide a priori information concerning biological activity related to the molecular structure of the substance under consideration. This information can be used to make either quantitative or

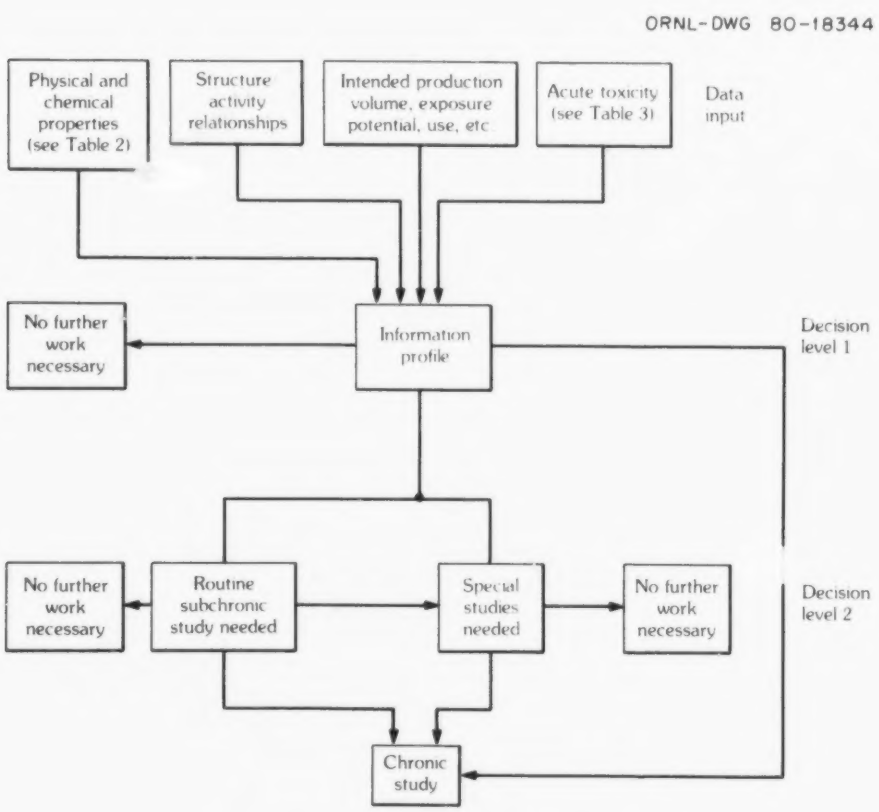

Fig. 1. Selection process for designing toxicity studies.

qualitative estimates of biological activity. The examination of structurally related chemicals of known toxicity which have physical-chemical properties (such as partition coefficients or Taft-Hammett constants for steric and electronic properties of substituents) similar to those of the test chemical may be useful in estimating the biological activity of that test chemical. This procedure may be of use either with a homologous series of chemicals or with chemicals that act on similar molecular targets. However, the necessary information for estimating biological activity may not always be available for many chemicals.

Some of the qualitative structure-activity correlations for the industrial chemicals of interest may relate to the carcinogenic potential rather than the general toxic or metabolic activity of the molecule. However, in the absence of experimental data, qualitative structure-activity correlations have some use in the prediction of potential target-organ toxicity or metabolic activity. Structure-activity correlations, therefore, may be of assistance in selecting toxicological parameters for evaluation.

In conclusion, much reliance in designing a subchronic study can and should be placed on the physical-chemical profile of the test compound and its intended use. Biological data from acute studies can also provide some guidance. Data on uptake, distribution, and elimination of a compound are dependent upon the availability of appropriate analytical methodology. Structure-activity relationships should be viewed and applied with caution and should never totally substitute for actual experimental data.

3. EXPERIMENTAL DESIGN

3.1 Exposure and Duration

Routes of entry for the chemicals of concern are inhalation, ingestion, and skin exposure. Ideally, the subchronic study should be conducted by the route of major concern relative to human exposure. It is recognized, however, that the subchronic study itself would generally be conducted by one route of administration and that multiple factors may be involved in the selection of that route.

An important concept in subchronic toxicity testing is that repeated doses of a substance, when adequate doses are administered, are likely to exert most of their potential long-term effects within 90 d. A lifetime "no-effect" dose can usually be predicted from such studies, excepting those effects referred to in the introduction. Weil and McCollister (1963) assessed the predictability of lifetime no-effect doses from subchronic data. Of 22 chemicals none had a long-term no-effect dose that was less than 1/20 of its 90-d no-effect dose. For most chemicals the ratio of the 90-d no-effect dose and the long-term no-effect dose was less than 10:1. The review of McNamara (1976) also supports these conclusions. Durations of less than 90-d have also been evaluated. Weil et al. (1969) found that even 7-d test results might be a sufficient indicator of long-term toxicity if used with a safety factor of appropriate magnitude. However, few references in the literature assess clinical and pathological effects for the shorter term with the 90-d studies.

In the majority of cases, peroral and inhalation studies should be accomplished in 3 months. In most inhalation studies comparable results will be obtained from either

the 5- or 7-d/week exposures. The inhalation exposure schedule should be 5 d/week, 6 h/d. For repeated dermal route studies, a maximum of 21 applications to rabbits on a 5-d/week basis is considered to be practical and of sufficient duration because of irritation and stress from repeated applications, daily restraint, and possible intercurrent infections.

3.2 Dose

To establish the extent of the toxic response, the highest dose should provide a distinct toxic effect but not lethality, whereas the lowest dose should produce no detectable toxic effect. To obtain maximum information on the dose response characteristics of the chemical, at least one intermediate dose is generally used.

3.2.1 Selection of doses

Guidance on the selection of doses for subchronic toxicity studies may be obtained from the results of prior acute and repeated high-dose studies. Assuming that the LD_{50} of the chemical has been established, some fraction of that dose may be assumed to be nonlethal but still toxic over the test period. This dose usually falls between 10 and 25% of the LD_{50}, depending largely on the slope of the acute dose response curve.

For chemicals having a tendency for bioaccumulation, the selection of dose levels is particularly difficult. Pharmacokinetic studies may assist in selecting the dose levels, because the elimination pattern may provide guidance on the extent of possible bioaccumulation. However, many of these factors may be unknown when the study is designed, so alternative approaches have been utilized for this purpose. Preliminary range-finding tests using 5 or more groups of rodents (perhaps as few as 2 or 3 animals per group) for a period of 3–4 weeks, with doses at twofold to fourfold intervals can be used to select dose levels.

The high-dose level should produce some significant toxicological effect without a high incidence of mortality that would prevent meaningful evaluation. The successful selection of a high dose that will produce these adverse but nonlethal effects is partially related to the results obtained from these studies.

3.2.2 Limitations of maximum levels

Occasionally, toxicological effects are not observed with a chemical even when very high doses are administered in a subchronic study. In such cases, there are maximum dose levels that need rarely be exceeded. In these studies, the maximum dose levels should not cause nutritional imbalance and/or affect the defense mechanisms. Maximum dose levels are considered to be 5% of the diet or 2.5 g/kg of body weight per day for peroral administration, 2 g/kg of body weight per day for dermal exposures, and 20 mg/m^3 (6 h/d) for inhalation of particulates, gases, and vapors.

Whenever these maximum dose levels are to be exceeded in actual use by humans, levels higher than these maxima should be used in subchronic studies, and care should be taken to avoid complications from artifacts that would prevent

meaningful utilization of the data. In some cases, factors such as low-vapor pressure, explosive limits, inability to achieve chamber concentrations, and insolubility and instability in vehicles may further limit the practical attainment of the desired high-dose levels. In a few cases, a chemical with low acute toxicity intended for low-volume productions may not be available in sufficient quantity to provide the desired high-dose levels in a subchronic study.

Because in this case the desirable objective of the subchronic toxicity test is to establish dose response patterns and no-observed adverse-effect levels, a minimum of three dose levels including at least one intermediate dose is generally used.

3.3 Species Selection

The animal species whose biological processing of the material being tested is most similar to man's should be used for subchronic experimentation. Because such information may be unavailable when subchronic studies are initiated, it is suggested that at least the rat, and probably the dog, be employed. Because in some instances the rat's sensitivity has been equal to or greater than the dog's, the rat has been at least as useful as the dog in detecting the lowest level at which an effect has been observed (Avidao 1978). However, the dog has shown greater sensitivity in other instances (Litchfield 1961; Homan 1972). In special circumstances, with a small number of chemicals, other species have been shown to be more sensitive than either the rat or the dog (Smith 1979). However, the use of additional species would often not appreciably affect the determination of a safe exposure level for man.

3.4 Number of Animals

The number of animals to employ in subchronic tests varies depending on the goals and needs of the investigator. The participants disagreed concerning the appropriate level of statistical analysis and the value of clinical chemistry/hematology tests. Therefore, two sets of animal numbers were suggested. If biochemistry and hematology tests are to be used as a major part of the assessment of toxic effects, then 20 rats per sex per level and 5 dogs per sex per level are necessary. If less use is made of clinical chemistry tests, then the numbers suggested are 10 rats per sex per level and 3 dogs per sex per level. For repeated skin application studies, 5 rabbits per sex per dosage level are recommended. Increasing the number of animals per level above that recommended will markedly increase the cost and decrease the efficient utilization of facilities and personnel; therefore, the choice of 3 dogs and/or 10 rats, or 5 dogs and/or 20 rats, should be justified by the investigator.

3.5 Age of Animals

In selecting the age of animals for use in a subchronic toxicity study, the ideal situation would have all age groups represented. Use of the neonate or fetus would allow the evaluation of toxic effects of chemicals on the earliest life stages. It would also be valuable to assess the toxic effects in older animals near the maximum life span. This would provide an estimation of the potential toxic effects for all ages of the human population. However, this ideal situation cannot easily be achieved because of such practical considerations as the needs to establish baseline data, allow for

acclimatization to the housing conditions, ensure the absence of nontreatment diseases, and even acquire test animals. All these require time and prevent the use of extremely young animals. Interference from normal aging effects and the difficulty in obtaining sufficient numbers of older animals of the same age make the use of older animals very difficult also. The subchronic test cannot be used to evaluate specific age-related effects either in the fetus, neonate, or older animals. Special studies would be required for such data. Therefore, in routine testing it is generally recommended that rodents be started on treatment as young as practically possible. This conceivably could be from 3 to 4 weeks of age; however, a period of 2 to 4 weeks usually is needed to assess the health and maturity of the animals and to perform pretest procedures. Thus, the earliest practical age to start rodents on test is 5 to 8 weeks.

For nonrodents, especially the dog, it is important that the animals have reached stabilized baseline levels for the biochemical/clinical parameters before they are started on test. Stablilization for many parameters, especially enzymes, often does not occur before 4 months of age in dogs because of variability in age of maturation and other reasons. For practical reasons and in keeping with the desirability of utilizing young adults, starting dogs on test at 4 to 10 months is preferable to using older animals.

Generally, young albino rabbits are used for dermal toxicity tests. It is suggested that rabbits weigh between 2 and 3 kg at the start of the study. Selection of other species or other age or weight ranges for dermal toxicity tests may be acceptable but should be justified.

4. EVALUATION OF TOXIC EFFECTS

4.1 Introduction

A series of interrelated tests are suggested for the general evaluation of biological systems, within practical constraints. In accordance with good laboratory practices, physical and clinical laboratory procedures will be used as indicators of toxicity, and tissue histopathology examinations will be performed on selected target organs. Additional clinical laboratory, and pathological tests may be employed if the indicator evaluations suggest abnormalities. The underlying purpose is to use cost-effective techniques to identify probable biological injury, which may then be examined in more detail. The following section discusses these tests and suggests a test design that provides a comprehensive evaluation.

The section is not meant to imply that only studies utilizing all these specific tests or combinations of these tests will be accepted by the EPA for TSCA regulation. The following discussions are included as examples of properly designed studies in which a complete array of toxicity evaluations are interwoven to achieve a satisfactory level of hazard assessment in a cost-effective manner. By indicating the perceived advantages of each test, the Clinical Evaluations and Pathology Committee hopes to provide a scientific basis for the selection of tests to include in a subchronic study design and yet avoid becoming rigid and overly complicated.

4.2 Clincial Observations and Evaluations

General clinical observations of experimental animals can include the periodic (preferably daily) evaluation of general health, physical activity, and behavior, in addition to observations on the function of specific systems, such as the cardiovascular, reproductive, eye and special senses, and central nervous system. In some instances, because of the animal species involved and the physical restraints of size and anatomy, certain suggested examinations and procedures will not be physically possible or practical (e.g., some procedures that are used in the dog may not be practical in the rat).

4.2.1 Physical

Experimental animals should be observed daily by trained and experienced laboratory and/or veterinary personnel (Balazs 1970; Benitz 1970; Janku and Krsiak 1973; National Academy of Sciences 1975; Prieur et al. 1973; Spurling 1977; Zbinden 1963). Physical observations include those of respiratory activity; appearance of the eye (e.g., cloudiness of cornea, conjunctivitis, discharge); nasal discharge; mucous membranes; skin color; and hair or coat appearance.

When practical, the heart and respiratory rates may be estimated by observation or by palpation upon routine handling (Benitz 1970). Quantitation of these rates may be performed when physical observations suggest a possibility of abnormality. Body temperatures may be taken if the need is indicated by the results of other observations, but in general, daily temperatures or exact pulse and respiration rates are too variable to be very useful in toxicological testing.

4.2.2 Health

Evaluation of the following parameters should be performed daily on a qualitative basis (Barnes and Denz 1954; Benitz 1970; Stevens 1977): appetite, presence and character of feces, water consumption when practical, presence of urine, and abnormal urinary consistency or color. When abnormalities are noted, urinary volume may be measured. Each animal should be weighed and its food consumption estimated at least once a week (Arnold et al. 1977; Barnes and Denz 1954; Benitz 1970; Smith 1950). A weekly determination of weight is suggested because body weight is often the only or earliest indication of toxic effects, and it ensures that every animal will have a weekly physical examination.

4.2.3 Cardiovascular

A specific daily or weekly evaluation of the cardiovascular system is generally not necessary, unless physical observations suggest abnormalities or unless chemicals suspected of affecting the cardiovascular system are involved. Then special examinations (e.g., electrocardiogram or blood pressure) may be performed (Benitz 1970).

Occasionally special monitoring of heart function will be an important part of toxicological studies, because at a specific concentration some chemicals will affect myocardial membranes enough to produce electrocardiographic changes. Among

laboratory animals the techniques of collecting and evaluating an electrocardiogram (EKG) is adequately developed only in the dog (Detweiler, Huben, and Patterson 1960; Detweiler and Patterson 1965). In addition to before dosing and at the end of the study, EKGs should be taken during the first week of the study because significant but transitory cardiotoxic effects may occur early in the dosing regimen. The frequency of further testing is determined by the necessity to detect any suspected change early and by the desirability of following the development of such cardiac alterations including any possible recovery. The time of day the EKG is taken should be determined in relationship to the metabolism and kinetics of the compound; generally, it should be taken at the time of the chemical's peak cardiac effect.

4.2.4 Reproductive

The most reliable early detector of an abnormality of the male reproductive system is histologic examination of the testes and epididymides, after proper tissue collection and fixation. When the prior history of the chemical suggests a potential effect on reproductive function, the male reproductive system may be monitored by a semen evaluation obtained by electroejaculation or another method. Semen samples may be evaluated for quantity and sperm motility, count, and morphology. This evaluation is not a routine procedure.

During clinical examinations of male dogs, palpation of the testicles for abnormal swelling and softness as well as the prominance and delineation of the epididymides may be useful in detecting toxic insult to these organs. Subchronic studies do not include an adequate detector of reproductive ability in the female; such detection would require a separate specific study.

4.2.5 Ocular

On a routine basis the sight of the animal may be evaluated by observing its usual movements within its housing area. Visual examination of the external eye is usually performed as part of the routine physical examination (Balazs 1970). If visual defects are suspected, further perception studies or more detailed ophthalmologic examinations should be performed. Biomicroscopic (slit lamp) examinations may be used for large animals (i.e., dogs and monkeys).

Ophthalmologic examinations are an essential part of subchronic toxicity studies (Benitz 1970; Grant 1974; Meier-Ruge 1977). They should be conducted on a representative number of the animals in the control and high-dose groups before the study and at its termination, because ocular lesions generally develop slowly and are seldom reversible (Grant 1974; Meier-Ruge 1977). If periodic or progressive changes are expected and if it is necessary to document the development of such changes, more frequent examinations may be performed. These examinations should be performed by a qualified person.

4.2.6 Behavioral

Evaluation of behavior has traditionally been incorporated into toxicology procedures (Balazs 1970; Benitz 1970; Janku and Krsiak 1973; Prieur et al. 1973; Zbinden 1963). Many of the observations can be done routinely during the physical

examination, which can include such tests as evaluation of the animal's attitudes (is it alert or lethargic?), postural activities (movement and carriage of portions of the body, limbs, and head), responsiveness to demand (ability to move when prodded), and general behavior (is it suddenly aggressive or depressed from previous observation?). Such observations may be easily performed by trained persons familiar with the animal's usual behavior.

Evaluation of hearing may be determined by the animal's carriage and movement of ears or by the introduction of unexpected external noise into the environment. Such evaluations are relatively arbitrary and have not been standardized.

4.2.7 Neurological

Specific neurological examination may be performed monthly or when indicated. In rodents, this evaluation may consist of balance beam or rotating-rod procedures; in dogs more detailed studies may be performed, such as electroencephalograms (EEGs) (DeLahunta 1977; Hoerlein 1971).

A detailed clinical and, when possible, instrumental neurological examination should be made if it becomes apparent that treatment-related signs of nervous system abnormalities are present. The objective of the test should be to determine the location of the lesions so that ultimately these areas can be evaluated by histopathologic or ultrastructural examination.

When neurological examinations suggest some abnormality, it is likely that continued exposure to the chemical compound in question could produce morphologic changes in the peripheral nerves or central nervous system. It may be appropriate in such cases to proceed with special additional studies at higher doses and more concentrated exposures to provide definitive morphologic-pathologic changes for better interpretation of these toxicologic effects.

4.2.8 Memory and learning

Memory and learning studies are recent additions to special toxicologic studies and are generally not included in routine subchronic studies. Memory and learning patterns of rodents and dogs may be tested and evaluated during and following exposure to chemicals. The procedures are currently limited to simplistic studies of primitive learning patterns or abilities to remember trained or conditioned activities. Mazes or pedal-pressing and reward trials may provide preliminary evidence of alterations in nervous system function.

4.3 Clinical Laboratory Tests

The laboratory procedures used for monitoring animals in subchronic toxicity experiments are usually tests that have been developed for diagnostic purposes. Tests developed for one species are usually appropriate for another but need to be modified because of quantitative differences. In monitoring animals in toxicological studies to detect early deviations from normal, absolute precision, accuracy, and sensitivity of test procedures are required.

The test selected should be appropriate for the animal species being utilized, be selected to monitor a particular major organ or to react to a broad range of biological

changes, and be the most sensitive in the normal range for that species. The test procedure should be sufficiently precise so as not to add to the total statistical variation of the results and allow comparison of results between laboratories.

4.3.1 Sampling frequency

The following suggestions are based on the use of the rat and the beagle dog as the test animals.

Because of the undesirable effects caused by frequent blood sampling, care should be taken not to collect at one time more than 10% of the animal's total blood volume. More than this will stress the capacity of the animal to regenerate lost red cells and would deplete the stored iron so that the data generated under these conditions would be compromised.

If possible all animals in the control and experimental groups should be sampled at least once before the dosing is initiated. Half of the rats in each group should then be sampled at one week, one month (and if the study warrants, every six to eight weeks thereafter), and at the termination of the study. Precautions should be instituted so that excessive sample removal does not affect body weight gain (Cardy and Warner 1979). The remaining half of each rat group should be sampled only at the study's termination. Because of larger blood volumes, all the dogs in each group should be sampled at each sampling period and termination. Because of the expenditure of resources and the energies devoted in these types of studies, prior consultation with biostatisticians is recommended.

4.3.2 Hematologic evaluations

General hemalologic examination consists of cellular evaluation and hemostatic function evaluation. This might include packed red cell (PCV), which is the most reliable method of all the erythroid measurements on peripheral blood (Linman 1966); hemoglobin concentration (Hb), which is an excellent screening test (Wintrobe et al. 1974), especially the cyanmethemoglobin method recommended by the International Committee for Standardization in Hematology (Lewis 1967); erythrocyte enumeration (RBC), which is necessary for assessment of erythrocyte numbers and may be required for the calculation of mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC), which can be derived either from manual or automated methods, which is remarkably constant throughout vertebrates, rarely exceeding 37 g/dl (Medway and Geraci 1964; Hawkey 1977), and which can also serve as a quality control measure verifying the compatibility of Hb and PCV; MCV, which is directly measured in some electronic particle counters or may be obtained from RBC and PCV values; total and differential white blood cell counts (WBC), including absolute values, which are basic parameters for leukocyte evaluation; blood smear examination including morphologic evaluation of erythrocytes, leukocytes, and platelets and of their relationship to the quantitative data (Archer 1977; Wintrobe et al. 1974; Cartwright 1968; Linman 1966); prothrombin time (PT) (in dog only), which is a nonspecific screening test measuring an overall functional ability of the extrinsic coagulation system; and activated partial thromboplastin time (APTT) (in dog only), which is a nonspecific screening test measuring an overall functional ability of the

intrinsic coagulation system. Mean corpuscular hemoglobin is not suggested because its usefulness may not be commensurate with the expenses involved in generating and storing data.

Special tests, in addition to the standard screening tests, may be done when quantitative parameters are different statistically from those of control values by ±2 standard deviations (or by other justifiable standards), or when qualitative assays or clinical signs suggest a treatment-related hematologic abnormality. Special tests may include but are not limited to reticulocyte count, platelet count (smear confirmation is always necessary), Heinz bodies detection, methemoglobin determination, glucose-6-phosphate dehydrogenase (G-6-P-D) estimation, pyruvate kinase (PK), platelet aggregation, erythrocyte fragility, and iron determination and stain.

At termination a routine hematologic study is to be performed. If a treatment-related hematotoxicity is present, a more complete hematologic evaluation including PT, APTT, and bone marrow smear should be performed, together with histopathologic examinations of the hematopoietic organs.

4.3.3 Clinical chemistry evaluation

The following is a list of commonly used clinical chemistry determinations which, used as a unit, can provide a fairly complete chemical analysis screen of toxicity (Kaneko and Cornelius 1970). However, it is not implied that these tests can totally substitute for the use of histopathology or that these tests are the only clinical studies that will be acceptable by the EPA. The intent of this discussion is to suggest a practical clinical chemistry screen that may be used with histopathology to evaluate the majority of the body systems.

- Glucose (fasting blood): Is the best single parameter of carbonhydrate metabolic status and pancreatic islet cell intergrity. It also reflects nutritional state and physiological stress. For example, it can reflect adrenal, thyroid, and, indirectly, pituitary function.
- Glutamic-oxaloacetic transaminase (GOT): Reflects injury to the skeletal muscle, cardiac muscle, and liver. Because it is found in high concentration in the RBC, it is also useful as an indicator of the quality of the blood sample.
- Glutamic-pyruvate transaminase (GPT): Reflects liver injury in carnivores and omnivores.
- Cholesterol: Reflects nutritional status, pancreatic islet cell damage (diabetes), thyroid function, and cholestatic liver injury. In this unit of 13 recommended tests, it is the best for reflecting thyroid function.
- Total protein: Reflects nutritional status, liver injury, intestinal injury, renal injury, and several other pathological states.
- Albumin: Reflects protein imbalances and is required for proper screening interpretation of the total protein.
- Globulin: Reflects the presence of some lymphoreticular tumors, acute inflammation, liver injury, chronic suppurative disease, renal disease, and several others. Globulin is obtained by difference between total protein and albumin. If the protein, albumin, or globulin falls outside two standard deviations from the mean or if other

statistical parameters indicate chemical related variations, the proteins should be electrophoresed to obtain accurate values for levels outside the normal range.

- Sodium: Permits evaluation of acid/base imbalances, adrenal function, and intestinal injury.
- Potassium: Permits evaluation of acid/base imbalances, adrenal function, and intestinal injury, but always used in conjunction with sodium.
- Alkaline phosphatase: May reflect bone injury, hepatocellular disease, and adrenal hyperfunction (dog).
- Calcium: Permits assessment of the status of bone and of nutrition.
- Phosphorus: Reflects bone injury, renal disease, and nutritional status.
- Blood urea nitrogen (BUN): Reflects renal injury, prerenal and postrenal azotemia and advanced liver disease.

All of the above tests can be performed with approximately 0.75 ml of serum, which in the normal animal may be obtained from 2.0 to 2.5 ml of whole blood. Because of the volume, the use of rats with body weight of less than 200 g is a limiting factor. Therefore, it may not be possible to do all the suggested tests at all the sampling intervals. However, all tests can be done on the blood collected at the termination of the study. The combination of tests to be performed should be justified by the investigator.

4.4 Special Organ Evaluation

When data indicate that a particular organ or organs are affected, certain additional tests may be suggested to specifically assess damage to that target organ. The merits of these tests should be properly evaluated. Tests would normally be similar for all species tested, such as the rat and the dog, if practical aspects make their use feasible. Pulmonary function tests are not specifically listed, because, except for inhalation toxicity studies, lung changes would not ordinarily occur; but where such damage might be present, pathological examination of lung tissue is the most appropriate means of determining its extent and significance. When extrapolating data to man, it is especially important to recognize the unique differences expected between various experimental animal species in their responses to chemicals as reflected in their organ function tests. It is necessary to have trained and experienced comparative pathologists and toxicologists available for such interpretation. The following discussion contains some suggestions for such assessments.

- Adrenal. In addition to sodium, potassium, and alkaline phosphatase, special tests to provide a definitive clinical laboratory evaluation of the adrenal gland are cortisol and the adrenocorticotrophic hormone (ACTH) response test.
- Bone. In addition to calcium, phosphorus, and alkaline phosphatase, a special test is parathormone. Other histopathologic approaches may be more cost-effective.
- Brain and nervous tissue. If clinical signs and results of other tests warrant, creatine phosphokinase (CPK) and isoenzymes may be determined, in addition to a study of cerebrospinal fluid (chemistry and cytology).
- Intestine. In addition to total protein, albumin, globulin, sodium, and potassium, special tests may include serum protein electrophoresis; amylase (dog); absorption

tests for fat, glucose, and xylose; serum chloride; total plasma carbon dioxide; and qualitative or quantitative tests for fecal fat, protein, and carbohydrate.

- Kidney. In addition to BUN, total proteins, albumin, and globulin, special tests may include creatinine; endogenous urea clearance; endogenous creatinine clearance; urinalysis; and serum protein electrophoresis.
- Liver. In addition to GOT, GPT, albumin, globulin, total protein, alkaline phosphatase (AP), and cholesterol, special tests may include serum protein electrophoresis, direct and indirect bilirubin, gamma gultamyl transferase (GGT), lactate dehydrogenase (LDH) and its isoenzymes, Bromsulphalein (BSP) retention, serum bile acids, and biopsy.
- Muscle. In addition to GOT, special tests may include CPK and isoenzyme, LDH and its isoenzymes, and biopsy.
- Heart. In addition to GOT, special tests may include CPK and and its isoenzymes and LDH and its isoenzymes.
- Exocrine pancreas. In addition to total protein, albumin, globulin, sodium, and potassium, special tests may include amylase and lipase; serum protein electrophoresis; fecal trypsin; absorption tests for fat, glucose, and xylose; and qualitative or quantitative tests for fecal fat, protein, or carbohydrate.
- Endocrine pancreas. In addition to glucose, sodium, and potassium, special tests may include intravenous glucose tolerance tests, insulin response to glucose loads, and urinalysis.
- Thyroid. Special tests may include triiodothyronine by radioimmunoassay (T_3), thyroxine by radioimmunoassay (T_4), and the thyrotropin response test.

4.5 Urinalysis Evaluation

Although most toxicology guidelines recommend urinalysis as a test parameter (Federal Register 1978; Food Safety Council 1978; Prieur et al. 1973; U.S. Environmental Protection Agency 1979), it was the consensus that routine and detailed evaluation of randomly selected urine samples normally has a minimal usefulness in subchronic studies. If any urinalysis is performed, it is suggested that nonrodents be used and rodents only when there is a need based on expected or observed toxicity. Among the parameters that could be assessed are color, specific gravity or osmolarity, pH, protein, glucose, ketones, formed elements (RBCs, WBCs, epithelial cells, etc.), crystalline and amorphous materials, and blood pigments.

4.6 Pathology

4.6.1 Gross examination

Qualified scientists should perform or personally supervise the necropsies, and/or be immediately available for consultation at the site. Other trained employees may assist in the necropsies.

Animals must be necropsied as soon as possible after death. If necropsy cannot be performed immediately after the animal is killed, or found dead, a trained technician should immediately refrigerate (but not freeze) the animal at temperatures low enough to minimize tissue autolysis (4–8°C). Animals found dead upon routine

clinical examination should be necropsied as soon as possible to salvage usable tissues. Animals found moribund should be sacrificed to assure the collection of usable tissues.

The gross examination should include an initial physical examination of the external surfaces and all orifices, followed by an examination of tissues and internal organs in situ. The examination should include the following: external and internal surfaces of all hollow organs; cranial cavity and external surfaces of the brain and spinal cord; nasal cavity and paranasal sinuses; neck with its associated organs and tissues; thoracic, abdominal, and pelvic cavities with their associated organs and tissues; and the musculoskeletal system including testing manual-breaking strength of a long bone or rib. (The latter procedure may be employed by the experienced pathologist with reliable results and is more practical than elaborate instrumental techniques.) In inhalation studies and where pulmonary damage is indicated, the lungs should be inflated with a fixative to allow for better gross examination and preservation. A minimum of 20% of the lungs from different groups should be so fixed, with 50–75% fixed, in special instances, by the intratracheal perfusion technique.

The weights of the liver, kidneys, uterus, prostate (dogs only), and seminal vesicles (except for dogs) should be recorded. In experiments involving chemicals with suspected hypertensive effects, the weight of the heart should be recorded. Also, in experiments in which the chemicals have a hormonal effect, the weights of the gonads and the adrenals should be recorded.

4.6.2 Tissue preservation

The prosector should immediately preserve all tissues and organs from all test animals in 10% buffered formalin or another recognized and accepted fixative appropriate for the specific tissue(s) (Luna 1968). Sections (blocks) from the following tissues from all test animals, regardless of their time of death, should be properly preserved and routinely examined microscopically:

- all gross lesions, with a margin of normal tissues;
- brain, minimum of one transverse section each from the forebrain, midbrain, and hindbrain;
- spinal cord, minimum of one section from the thoracic region;
- eye and attached optic nerve;
- a major salivary gland;
- thymus;
- thyroid;
- heart, two sections, one through a left papillary muscle and one through a coronary artery;
- aorta;
- one lung with a major bronchus;
- stomach, fundic and pyloric regions;
- small intestine, three levels;
- large intestine, one level each of cecum and colon;
- adrenal gland;
- pancreas;

- liver, one lobe (a minimum of one left and one right lobe when gross lesions or other evidence of hepatotoxicity are present);
- gallbladder, if present;
- spleen;
- kidney;
- urinary bladder;
- mesenteric lymph node, plus tracheobronchial lymph nodes in inhalation studies and regional lymph nodes in dermal toxicity studies;
- bone, including marrow;
- skin, only required in dermal toxicity studies and should be selected from skin painting sites and normal areas;
- skeletal muscle, selected if there are clinical signs, gross lesions, or changes in appropriate serum enzymes (contraction artifacts should be prevented and enzyme histochemistry used when fiber types need to be identified); and
- reproductive organs, testes, and epididymides or uterus and ovaries.

The testes and epididymides, sectioned into 0.5-cm slices, should be preserved in Bouin's solution or other appropriate fixative to ensure a high quality histologic section. For detecting impairment of fertility in the male, this is the only method that is routinely done in these end-point toxicity studies. Based on experience, or scientific or practical judgments, other tissues may be preserved for later study.

4.6.3 Justification of selection of tissues for histopathological examinations

Histopathological examination of tissues is expensive, and in some organs it is not the most reliable or sensitive detector of damage (Smith, Jones, and Hunt 1972). Therefore, we have attempted to monitor changes in organs by those tests that are most sensitive and least expensive. Microscopic examination of some tissues included in other guidelines have been omitted from routine examination here when there is no gross lesion, no change in appropriate clinicopathologial parameters, or no evidence of toxicity from previous studies. However, monitoring of these organs has been done by other means (e.g., weighing organs, clinical pathology, or necropsy observations), although histopathologic examination may still be required in some studies. Except where observations indicate, the histopathological studies should be conducted on control and high-dose groups only.

For paired organs, it is necessary to examine only one if both are grossly normal because exposure is via the bloodstream and thus change is assumed to be bilateral. Only one lobe of the liver is sampled, if normal. If lesions are present, both a right and a left lobe should be sampled. Since portal streaming occurs in some species, an orally ingested toxicant could affect one lobe more than another (Popper and Schaffner 1974). For instance, toxicants absorbed from the small intestine could affect the right lobes more severely.

Parathyroid function is usually monitored by checking the breaking strength of bones at necropsy, the appropriate clinical pathology parameters (e.g., serum calcium), and, in dogs, the size of the gland at necropsy. Because lesions in grossly normal parathyroids are infrequently detected in Hematoxylin and Eosin-stained (H&E) sections (Robbins 1967), histopathological examinations of the parathyroids will not be done routinely.

The trachea need not be examined microscopically in oral or dermal studies, because they are unlikely to be exposed to significiant concentrations of the test material. If material is regurgitated and aspirated into the trachea and lungs, any changes would be detected in the sections of bronchi and lungs. The trachea should be examined at necropsy.

The esophagus is not routinely examined microscopically, because it has only transitory contact with ingested material. It is examined at necropsy and sectioned if there is a gross lesion. Irritant chemicals would produce gross lesions and/or clinical signs.

The changes in weight of the uterus and the prostate-seminal vesicle are monitored, because the weight changes are a more sensitive and earlier indicator than microscopic examination. Early hyperplastic changes in prostates are especially difficult to confirm by histopathological examination alone.

No separate collection of small arteries is required, as they will be examined in numerous other tissues.

Routine histologic examination of apparently normal skin is unlikely to reveal a lesion. Any skin lesion should be sectioned. The mammary glands can be examined clinically and sectioned if any alterations in size or texture are detected during the experiment or at necropsy.

Bone marrow function is monitored routinely by complete blood count (CBC). Hematopoietic function is routinely monitored by CBC examinations and, when necessary, bone marrow smears. At necropsy histologic examinations are made of sections of bone marrow, spleen, lymph node, and thymus.

Histopathological examination of the H&E-stained sections of pituitary is not likely to reveal treatment-related changes in a subchronic study. Where there is clinical or other indication of pituitary dysfunction, serial sections and appropriate special stains (e.g., modified trichrome stains) should be prepared (Luna 1968).

Routine examinations of the liver, gallbladder, kidney, urinary bladder, and salivary gland are included, because these organs excrete foreign chemicals that are absorbed into the body.

Treatment-related lesions in the heart are infrequent in experimental animals, but because of the frequency of lesions in the human heart and aorta, these are routinely sectioned. However, examination of these organs may not contribute significantly, and this routine sectioning should be reevaluated when adequate data are available

Visual function can be monitored through changes in animal behavior and by ophthalmological examinations. However, one eye and its attached optic nerve will be routinely cross- and longitudinally sectioned because of the possibility that animals may have suffered visual impairment that escaped clinical detection.

4.6.4 Special considerations

Postmortem artifacts in muscle contraction may be prevented by clamping or by allowing the muscle energy stores to be depleted before fixation (Adams, Denny-Brown, and Pearson 1962; Price et al. 1965). If lesions are suspected, then skeletal muscles, particularly the weight-bearing muscles of the pelvic and pectoral girdles (Bradley 1978), should be examined at necropsy. During trimming, each muscle should be identified and cut in longitudinal and transverse sections, and enzyme

histochemistry (Dubowitz and Brooke 1973) and electronmicroscopy may be necessary to characterize muscle lesions in more detail (e.g., to determine fiber types involved).

In an inhalation study, multiple sections of the upper respiratory tract, external nares, nasal turbinates, nasal cavity, paranasal sinuses, hypopharynxlarynx, and tracheobronchial lymph nodes should be sectioned.

4.6.5 Central nervous system

Successful detection of lesions in the nervous system depends on examination of the correct sites. Because examination of the nervous system is difficult and frequently impossible, a thorough clinical examination may assist in characterizing the alterations.

Where lesions of the central nervous system and peripheral nervous system are suspected, transverse sections of the brain and cord are examined. Cord segments at the levels of C_2, C_8, T_7, and L_6 or L_7 should be sampled. A minimum of six brain and four cord segments is preferred for the initial examination of the central nervous system. The nature and location of the lesions should be characterized.

For microscopic examination, brain and spinal cord sections should be chosen based on clinical signs. In special cases, brain and cord may be fixed in situ by systemic perfusion with 4% paraformaldehyde followed by 5% glutaraldehyde. In dogs with clinical signs but nonlocalized lesions, the following transverse sections of brain should be considered for selection: corpus striatum, optic chiasma and optic tracts, mammillary bodies and hippocampus, rostral colliculus, caudal colliculus, cerebellum and medulla at the level of the pons, cerebellum and medulla at the level of the facial nerve nucleus, medulla at the level of the vestibular nuclei, and medulla at the obex.

Characterization of lesions should not depend on H&E-stained sections alone. Selected electronmicroscopy, as well as special stains such as Holmes for normal axons, Guillery for degenerating axons, Luxol fast blue for normal myelin, Glee's modification of Marchi for degenerating myelin, osmium tetroxide staining of teased peripheral nerves, and Holzer's stain for glial fibers may be used where appropriate.

4.7 Analysis and Presentation of Test Results

The report of the subchronic study should contain a summary and analysis of the data and a statement of the conclusions drawn from the analysis. The summary should highlight any and all toxicologically significant data or observations and give the investigator's opinion as to the mechanism of the action of the toxicant, the target organ or organs, and the sequence of pathological changes.

4.8 Usefulness and Correlation of Test Results

This section on the evaluation of toxic effects describes the usefulness and correlation of sets of general observations, clinical laboratory tests, and pathology examinations to be routinely evaluated in a subchronic toxicity study. It is suggested that additional tests and examinations may be performed when appropriate. Table 4 shows how these three sets complement each other and allow evaluation of organs or organ system(s) with the most appropriate and economical methods now available.

Observations in subchronic tests that might trigger further testing were considered. However, the variety of biological changes produced by chemicals is so great and variable that no specific recommendations could be made.

Table 4. General observations, clinical laboratory tests, and pathology examinations that may be used in subchronic toxicity studies

Organ or organ system	General observations	Clinical laboratory tests on blood	Pathology examination[a]
Liver	Discoloration of mucous membranes, edema, ascites	Glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase, alkaline phosphatase (AP), cholesterol, total protein, albumin, globulin	Liver[b]
Gastrointestinal (GI) system	Diarrhea, vomit, stool, appetite	Total protein, albumin, globulin, sodium (Na), potassium (K)	Stomach, GI tract, gall bladder (if present), salivary gland, pancreas
Urinary system	Urine volume, consistency, color	Blood urea nitrogen, total protein, albumin, globulin	Kidney and urinary bladder[b]
Hematopoietic hemostatic system	Discoloration of mucous membranes, lethargy, weakness	Packed red cell volume, hemoglobin, erythrocyte count, total and differential leukocyte count, thrombocyte count, blood smear, prothrombin time, activated partial thromboplastin time	Spleen, thymus, mesenteric lymph nodes, bone marrow smear and section
Nervous system	Posture, movements, responses, behavior		Brain, spinal cord, and sciatic nerve
Eye	Appearance, discharge, ophthalmologic examination		Eye and optic nerves
Respiratory system	Rate, coughing, nasal discharge	Total protein, albumin, globulin	One lung with a major bronchus
Endocrine system	Skin, hair coat, body weight, urine and stool characteristics	Glucose, Na, K, AP (dog), cholesterol	Thyroid, adrenal, pancreas
Reproductive system	Appearance and palpation of external reproductive organs		Testes and epididymis or ovaries, Uterus or prostate and seminal vesicles[b]
Skeletal system	Growth, deformation, lameness	Calcium, phosphorous, AP	Bone and breakage strength
Cardiovascular system	Rate and characteristic of pulse, rhythm, edema, ascites	GOT	Heart,[b] aorta, small arteries in other tissues
Skin	Color, appearance, odor, hair coat	Total protein, albumin, globulin	Only in dermal studies
Muscle	Size, weakness, wasting, decreased activity	GOT, creatine phosphokinase	Only if indicated by observations, clinical chemistry or gross lesions

[a] All animals should undergo thorough gross examination; organs or tissues listed should be examined microscopically.
[b] These organs should also be weighed.

5. CRITERIA FOR DATA EXTRAPOLATIONS FROM ONE ROUTE OF ADMINISTRATION TO ANOTHER

5.1 Introduction

In the evaluation process mandated by the Toxic Substances Control Act (TSCA), there may be cases either in which existing data have been obtained by a route of administration other than the primary exposure route or in which the primary route is impractical. Such situations establish a need for being able to extrapolate and apply data obtained by one route of administration to another route of administration. The objectives of this committee were to consider the feasibility and acceptability of making route-to-route extrapolations and to define the criteria to be considered and/or employed in making such extrapolations.

5.2 Review of Data

In general there is available in the literature little information about assessing the toxicity of individual compounds as a function of the route of administration. For comparative purposes, it is necessary that the same compound be compared by different routes in the same species and that the same end point be observed. A computer search of the literature, covering more than 30,000 potentially appropriate titles and abstracts, did not reveal many situations where suitable data were available. The data that was available was used mainly to develop the following discussions and recommendations.

Kast et al. (1975*a*) compared oral vs intravenous vs inhalation for the beta-sympathomimetic drug Fenoterol HBr. The range of inhalation doses per day was estimated to be 0.01–1.0 mg/kg (mg per kg of body weight), whereas the estimated range of the oral dose (gavage) per day was 15–1500 mg/kg. Studies by Case, Smith, and Nelson (1975) compared acute toxicity of the nonnarcotic analgesic Nefopam by measuring the LD_{50} as a function of route (Table 5). All three species showed that the oral route is about half as toxic as the intravenous or the intramuscular. They also tested the compound subchronically, but the doses were too low to observe effects.

Several reports point out the difficulties and pitfalls of attempting to extrapolate from one route to another. Casarrett (1975) states:

> As one views the duration of ... individual exposures in a chronic series, the importance of other considerations becomes apparent. The duration of an exposure for gases and vapors administered by the pulmonary route would ordinarily be longer than by other parenteral routes. Administration via the lung or gastrointestinal tract results in a delay, which may be small or great, in the absorption and distribution to active sites and will have a bearing on the effects observed.
>
> Parenteral administration also results in varying routes of absorption and distribution. The intravenous route results in a virtually instantaneous accessibility to the circulating blood. ... The order of rapidity of absorption for soluble substances may be represented as an approximate range from fastest to slowest as follows: intravenous, inhalation, intraperitoneal, subcutaneous and intramuscular, intradermal, oral, and topical. (p.13)

Loomis (1974) discusses the influence of the route of administration on toxicity. He notes examples where the LD_{50} is dependent, independent, or partially dependent on

Table 5. Acute toxicity of nefopam in three species

Route	LD_{50} (mg/kg)[a]	LD_{50} (95% confidence limits)
	Mouse	
Intravenous	45.5	36.5-54.3
Intramuscular	52.9	41.3-67.7
Oral	119	101-135
	Rat	
Intravenous	28	25.0-31.4
Intramuscular	56.9	47.4-68.3
Oral	178	146-217
	Dog	
Intravenous	~20	
Intramuscular	~30	
Oral	>100< 200	

[a]Mg per kg of body weight.
Source: Adapted from Case, Smith, and Nelson, 1975.

the route of administration (Table 6). He further suggests that comparative LD_{50}'s of a compound given by different routes may allow prediction of information concerning translocation, deposition, inactivation, or site of excretion of the compound in question. He does not, however, suggest a rationale for estimation of toxicity of a compound given by one route when information is available about the toxicity when given by another route. Limited data from studies not appearing in the available literature suggest that route-to-route correlations and subsequent estimations are possible for some compounds but not for others.

Table 7 compares the results of two 90-d toxicity studies in rats with vinylidene chloride (VDC). In both studies the primary toxic sign, degenerative fatty liver changes, observed at daily doses of 28 mg/kg in the drinking water study and 42 mg/kg in the inhalation study, was described as being slightly more severe by the inhalation route. The correlation was also observed when similar experiments were performed on fasted and nonfasted rats, even though toxicity was more severe in fasted than in nonfasted rats. It appears that with this approach, which uses breathing rates of rats and chamber concentrations and assumes 100% absorption, there is a positive correlation between the daily dose on a mg/kg basis and the observed toxicity for two routes of exposure.

Table 8 compares the toxicity of perchloroethylene (PERC) administered in rats by either gavage or inhalation. In the gavage study PERC was administered in a corn oil solution daily at a dose of 500 or 1000 mg/kg for two years. In the inhalation study rats were exposed to 10 or 600 ppm of PERC for one year followed by one year of observation without exposure. Both studies reported that the only toxicity observed was the early onset of mortality in males due to chronic renal disease. (Males of this strain of rat have a naturally high rate of spontaneous chronic renal disease.) Calculation indicates that doses of 10 and 600 ppm for 6 h/d approximate daily doses

Table 6. Effects of administration of compounds in which lethal toxicity is independent of (isoniazid), partially dependent on (DFP[a] and pentobarbital), and completely dependent on (procaine) route of administration

	Procaine[b]		Isoniazid[b]		DFP		Pentobarbital[b]	
	Mouse		Mouse		Rabbit		Mouse	
Route of administration	LD_{50} (mg/kg)[c]	Ratio (x/iv)[d]	LD_{50} (mg/kg)	Ratio (x/iv)	LD_{50} (mg/kg)	Ratio (x/iv)	LD_{50} (kg/kg)	Ratio (x/iv)
Intravenous	45	1	153	1.0	0.34	1.0	80	1.0
Intraperitoneal	230	5	132	0.9	1.00	2.9	130	1.6
Intramuscular	630	14	140	0.9	0.85	2.5	124	1.5
Subcutaneous	800	18	160	1.0	1.00	2.9	130[e]	1.6
Oral	500	11	142	0.9	4.9	11.7–26.5	280	3.5

[a] Diisopropylfluorophosphate. Data from *Handbook of Toxicology*, vol. 1, *Acute Toxicities*, W. S. Spector, ed., W. B. Saunders Co., Philadelphia, 1956.

[b] Data from C. D. Barnes and L. G. Eltherington, *Drug Dosage in Laboratory Animals*, University of California Press, Berkeley, Calif., 1964.

[c] Mg per kg of body weight.

[d] Ratio between the particular route (x) and the intravenous (iv) route used as baseline.

[e] Personal data from author's laboratory.

Source: Adapted from Loomis, 1974.

Table 7. Comparative results of 90-d toxicity studies in rats administered vinylidene chloride by drinking water or inhalation

	Drinking water			Inhalation[a]	
Concentration (ppm)	50	100	200	25	75
Daily dose (mg/kg)[a] Mean dose	5–12 ~8	8–20 ~14	16–40 ~28	14[b]	42[c]
Degenerative fatty liver	Absent	Absent	Present	Absent	Present[d]

[a] Exposure for 6 h/d, 5 d/week.
[b] Mg per kg of body weight.
[c] Calculated using breathing rate of rat and the concentration in air.
[d] Slightly more pronounced than that observed in the drinking water study.
Source: W. Braun, Dow Chemical Co. (unpublished data).

Table 8. Comparative toxicity in rats administered perchloroethylene by gavage or inhalation

	Oral gavage		Inhalation	
Concentration (ppm)			10	600
Daily dose (mg/kg)[a]	500	1000	20	1200
Early mortality males (chronic renal disease)	Absent	Present	Absent	Present

[a] Mg per kg of body weight.
Source: Pegg et al., 1979.

of 200 and 1200 mg/kg respectively. When this approach is used, there again appears to be a correlation between the observed toxicity studies and the daily doses expressed on a mg/kg basis.

These apparent correlations of dose with observed toxicity for two different routes of exposure should not be taken as a general correlation that will hold in all cases. The correlation noted for PERC is most probably reasonable and is easily explained, because Pegg et al. (1979) have demonstrated that the pharmacokinetics and metabolism of PERC are independent of the route of exposure. However, when pharmacokinetics and metabolism are dependent upon route of exposure, correlation with effect may not be apparent and extrapolation becomes more difficult.

Table 9 summarizes the results from two chronic toxicity studies in rats with 1, 4-dioxane administered either via inhalation or drinking water. Previously mentioned dose calculations indicate no evidence of correlation between dose and observed toxicity. Young, Braun, and Gehring (1978) demonstrated that blood concentrations of 1, 4-dioxane were comparable for estimated equivalent doses by either drinking water or inhalation exposure. Liver concentrations of 1, 4-dioxane, however, were much higher after oral administration.

One of the factors that could form a potential basis for extrapolation, with some degree of confidence from one route to another would be an equivalence in peripheral

Table 9. Summary of results from two chronic toxicity studies in rats with 1,4-dioxane

	Drinking water		Inhalation
Concentration (ppm)			111
Daily dose (mg/kg)[a]	100	1000	105
Toxicity	Liver toxicity	Tumors	No toxicity in liver or other organs

[a] Mg per kg of body weight.
Source: Adapted from Young, Braun, and Gehring, 1978.

blood concentrations. However, this principle would not apply in situations like the following.

- The organ of entry is the target organ. If an inhaled compound produces an effect on the lung, it would be unlikely (but not impossible, because there are systemic lung toxicants) that the effect would be produced by ingestion. Similarly, if ingested materials cause stomach damage it would be difficult to extrapolate that effect to a different route.
- The organ of entry to another organ or system is without the benefit of detoxication. Two examples are apparent. The effects of inhaled materials on the heart and the effect of ingested materials directly on the liver. In these examples the heart and liver are exposed to the material directly as it comes from the organ of entry. The concentration in the pulmonary and hepatic veins may be far greater than that in the peripheral blood, and thus the potential for effect is much greater for the heart and liver.
- The compound is highly reactive. In this case administration by various routes may result in totally different target organs or metabolites.

Generally, it is reasonable to assume that once a material enters the circulatory system, its subsequent behavior is independent of route of administration. Thus, if the blood concentration is known for two routes of administration as a function of time and dose (exposure) and if the toxic effects via one route are known, prediction of toxicity by the other route is possible. In the absence of either blood kinetic information or exposure-blood level information, the prediction of toxicity by a different route will be highly uncertain.

In summary, few studies allow for estimations of the type mentioned above, and many studies indicate that extrapolation from one route to another would be impossible. In the absence of any other information and where some estimations are needed, the approach as outlined by Stokinger and Woodward (1958) could be followed.

This approach used retrospective studies and standards for extrapolating data from various routes of exposure to develop drinking water standards. Because much work had been done on the classes of chemicals they were dealing with, the authors relied heavily on established standards such as Threshold Limit Values for industrial

chemicals in air, federal tolerances for pesticide residues, toxicological classifications by type of compound, and toxicity data from nutritional and other studies.

The authors used similar adaptation techniques for data generated in animal tests by various routes. The following summarizes the basics of the extrapolation process.

- For data from inhalation studies, LD_{50} or related values should be converted to a common dosage unit (i.e., mg/kg or mg/kg per day) through the use of standard respiratory parameters for the subject species over the exposure period. This adaptation must include the development of absorption factors that estimate the amount of test material absorbed into the blood stream. Use of these factors introduces greater uncertainty into establishing uniform exposure situations, but data from kinetic studies and physical characterization of the test material may diminish the uncertainty somewhat.
- For ingestion data, toxic values should be expressed in standard units suitable for conversion, such as those listed above. An absorption factor must again be developed by using whatever data is available to diminish the inherent uncertainty.
- For dermal data, suitable dosage units can be calculated (but exposure times must be carefully studied in light of the test design being reviewed).
- For parenteral data, similar standards can be developed, but absorption factors, depending on the materials being studied, may be made with greater confidence.

After conversion of known data to uniform dosage units, it follows that the reverse process is used to calculate exposure concentrations, applied doses, etc.

Thus, the only available reference that suggests a reasonable approach to the problem of extrapolation from one route to another appears to be Stokinger and Woodward. In essence, it suggests a considered judgment of all available toxicological data in attempts to carry out such an extrapolation.

5.3 Factors Affecting Extrapolation of Data Obtained by One Route of Exposure to Another

The effect of route of administration on absorption, distribution, metabolism, excretion, and potential toxic response is well known. However, the committee felt that a brief review of the subject was worth including.

"As a general rule... absorption of chemicals will be most rapid when given by inhalation, less rapid when given by gavage, and slowest with dermal application" (World Health Organization 1978, p. 68). The order is affected by the role of physico-chemical factors in absorption. In general the skin is not highly permeable to most chemicals and acts as a reasonable barrier against environmental chemicals. Systemic injury and death can result, however, from some chemicals which pass through the skin with relative ease. Klaasen (1975) points out that for most materials a large number of dermal cell layers act as barriers to absorption through the intact skin in comparison to absorption through the lung and gastrointestinal tract, which represent only a one- or two-cell layer barrier. Because the intact stratum corneum represents a major barrier, chemicals that injure this structure may be more rapidly absorbed.

Substances present in the lung or gastrointestinal tract have in common the fact that they are essentially outside the body and produce only localized surface effects until they are absorbed into the bloodstream. Chemicals absorbed from the lung are carried directly to the heart and then into the systemic circulation. In contrast, most compounds absorbed from the gastrointestinal tract are carried directly to the liver, which in many instances may be a direct target organ. Gastrointestinal absorption, in contrast to lung absorption, is complicated by several functions of the liver. Because of its size and enzymatic activity, the liver is the major site of metabolism of most xenobiotics and is often the target organ of the metabolites formed within its cells. A variety of chemicals may be excreted through the bile either unchanged or in the form of metabolites. Once returned to the gastrointestinal tract, these compounds may be reabsorbed to produce an additional toxic impact on the liver.

In summary, the initial impact of chemicals absorbed from the lung is considerably different from effects following absorption from the gastrointestinal tract. Thus the portal of entry may play an important role in the toxic response, particularly where the immediate organ involved (lung, liver, or skin) is the target organ. Target organs different from the portal of entry and exposed strictly from the systemic circulation would be expected to respond directly to comparable blood levels of compound or metabolites regardless of the route of entry.

Physico-chemical properties of the chemical may influence the extent of systemic absorption by various routes of exposure and thereby the development of the test procedures. This is discussed in earlier sections.

5.4 Assumptions and Criteria for Extrapolation

The following assumptions were discussed by the committee.

- Given quantitative and temporal equivalency of circulating blood levels of a chemical, it is reasonable to assume that systemic effects will be comparable regardless of the route of administration.
- In making route-to-route extrapolations, only systemic effects other than those occurring at the portal of entry should be considered or compared.
- Effects at the portal of entry should be considered and evaluated separately.

When results of a subchronic toxicity test are to be considered as definitive, the committee recommends that a study by the primary human exposure route be conducted. Under the following conditions, study by the primary route is totally impractical.

- In certain cases the vapor pressure of a compound is low, yet exposure is to the vapor. It would be difficult to produce vapor concentrations high enough to induce toxicity in animals. Particulate aerosols could be produced, but exposure to these would not adequately represent exposure to the vapor. In this case exposure by ingestion might be entirely appropriate.
- Certain industrial dusts are produced in a unique manner (e.g., fiber coating dust in making fibers). In this case, ingestion may be the only feasible route of administration.

- Vapors and organic dusts are possible explosives. While every effort should be made to conduct vapor exposures via inhalation, an inhalation study may not always be possible. The hazards of dust aerosols could preclude inhalation studies.
- Cost and availability of compounds may at times preclude inhalation studies, particularly those involving whole body exposure, which are very wasteful of test materials, and those using developmental products.

The Route-to-Route Extrapolation Committee considered the extrapolation of subchronic data obtained by ingestion of chemicals in the diet to predict effects of inhalation exposure to be the most likely to be employed and the most scientifically acceptable extrapolation. Inhalation-to-ingestion extrapolations are probably feasible and scientifically reasonable, but will be rarely needed. Extrapolation and estimation of systemic effects via the inhalation or oral route from data obtained in dermal studies was considered to be feasible but not very desirable because of a lack of defined methodology for conducting dermal subchronic studies. Extrapolation to the dermal route was not considered to be a probable need.

The validity of any extrapolation should be evaluated when toxic signs developed in testing by one route indicate that changes might occur in parameters that affect the absorption, distribution, metabolism, etc., by the untested route.

The committee recognized the need for separate consideration of extrapolation criteria to be applied in situations where data are in existence (retrospective analysis and extrapolation) and where data are to be developed (prospective analysis and extrapolation).

5.5 Retrospective Analysis and Extrapolation

Retrospective analysis involves the gathering and evaluation of all available data on the compound of interest. The objectives of the analysis are (a) to render a judgment as to whether the available data are adequate for making an extrapolation without developing additional data and (b) to determine what additional data are needed if existing data are inadequate. In retrospective analyses, the existing data are likely to range from no data to data from a considerable number of studies that relate to numerous toxicological responses.

The following are suggested as guidelines for data evaluation.

- The subchronic study (or studies) from which data are to be extrapolated must have been adequately designed and conducted to allow for proper application of extrapolated results. For example, a subchronic study designed and conducted to evaluate compound-related effects in only one tissue or organ would not be considered adequate for risk assessment purposes.
- Data from studies on species or animal strains different from those used in the subchronic study are not acceptable for extrapolation purposes.
- At least one study that assesses compound-related effects at the portal or organ of entry by the route to which extrapolation is to be made is strongly recommended.
- At least one study of the same type and duration (e.g., LD_{50}, LC_{50}, EC_{50}, etc.) and utilizing the same end point(s) by each of the two applicable routes of administration is essential.

- Studies that make route-to-route comparisons of such things as pharmacokinetics and metabolism are highly desirable. At a minimum, comparative blood level data should be available if any degree of confidence is attached to extrapolated results. Data on the other parameters can substantially increase confidence in extrapolated results.
- Comparative studies that assess biochemical, hematological, clinical, pathological, or other parameters can also increase the degree of confidence.

Under conditions when it would not be practical to perform a study by the untested route, the procedure presented for prospective analyses is recommended.

5.6 Prospective Analysis and Extrapolation

Prospective analysis, as envisioned by the committee, is limited to those situations in which exposure by the primary route of anticipated human exposure is impractical and in which either no data exist or existing data are inadequate for extrapolation purposes.

In these situations, the investigator has the advantage of being able to plan carefully all studies involving extrapolation.

For prospective extrapolations, the committee considers the following as being representative of ideal requirements:

- data that demonstrate a similarity in acute and/or subchronic response(s) (including lethality) to the test material by both routes of administration;
- data that demonstrate equivalency in dose as evidenced by blood levels, total body burden, or similar parameters;
- data that reasonably support the conclusion or assumption that pharmacokinetics are independent of route of administration;
- data that reasonably support the conclusion that effects at the organ or portal of entry by the tested route would or would not be anticipated to occur as a result of exposure by the untested route; and
- data that address the possibility of effects at the portal of entry for the untested route.

Given the knowledge that development of all ideal data will not be feasible, the committee considers certain combinations of these data to be most desirable.

The best situation for extrapolation would permit a 14-d study by both routes of administration. Such a study would serve to establish the target organ(s) and to demonstrate direct effects on the organ of entry. If the same toxicologic end point were observed after dosing by both routes, extrapolation from one route to another after a 90-d study could be carried out with reasonable confidence. In the absence of a common effect, the concentration of the test material in blood during the 14-d studies could be used to predict results from an alternative route. The error associated with this extrapolation would be somewhat greater than in the previous case.

If 14-d comparisons cannot be performed, comparisons of LD_{50} data between each of the routes could be generated. This would serve to equate the doses by the two routes. However, because the cause of death after acute studies may not be related to subchronic toxicity, this approach would be less desirable.

If data comparing toxicity as a function of route cannot be obtained, the extrapolation is more difficult. However, when a need exists, the following information would be useful:

- physical and chemical properties (solubility in water, acids, saline, octanol-water distribution coefficient, particle size in the atmosphere, and pH);
- pulmonary toxicity by intratracheal routes;
- any information on distribution, metabolism, retention, and excretion;
- target organs; and
- in vitro studies.

5.7 Summary and Conclusions

- any extrapolation of toxic dosage levels from one route to another must be considered in the light of the associated level of uncertainty. Situations exist wherein valid extrapolations cannot be made.
- The justification for extrapolating data rather than performing the subchronic test should be based on scientific rationale rather than on economics alone.
- The literature base for making valid extrapolations is almost nonexistent, although much data may exist either in unpublished form or in a form not readily identifiable through normal search procedures.
- Most extrapolations would be made using data from subchronic oral dosing to predict inhalation effects, although the reverse may also occur. Some extrapolations may be made using acute and subchronic dermal studies to predict toxicity by other routes, but the reverse is not practical.
- All available sources of information should be sought out to minimize the uncertainty of such extrapolations. Data from acute, short term, and subchronic studies from all available routes, human exposure, and kinetic and physical-chemical input will be needed.
- Extrapolations of this type should be performed by qualified scientists.

6. LIMITATIONS OF ACUTE AND SUBCHRONIC TOXICITY STUDIES

6.1 Introduction

The rationale for the use of the results of toxicity studies in animals and suggested limitations of these studies are summarized in the following paragraph taken from the World Health Organization (1978) document:

> In many cases, studies with laboratory animals make it possible to predict the toxic effects of chemicals in man. However, it is important to realize that experimental animal models have their limitations, and that the accuracy and reliability of a quantitative prediction of toxicity in man depend on a number of conditions, such as choice of animal species, design of the experiments, and methods of extrapolation of animal data to man. (p. 35)

Because there is always some uncertainty in extrapolating the results of toxicity studies in animals to man, a safety factor is introduced to compensate for this

uncertainty. The World Health Organization document addressed this point as follows:

> In general, the size of the safety factor will depend on (a) the nature of the toxic effects, (b) the size and type of population to be protected, and (c) the quality (and quantity) of toxicological information available. (p. 38)

6.2 Acute Toxicity

Acute toxicity studies (oral, intravenous, dermal, ocular, inhalation, etc.) demonstrate biological potency. On this basis an estimate of hazard may be predictive for a single, accidental human exposure. This extrapolation is not always valid, but interpretation of the results may be strengthened by comparison with results for similar chemicals.

6.3 Interpretation and Limitations of Acute Toxicity Studies

The single-dose oral and parenteral (intraperitoneal, intravenous, inhalation, ocular and/or dermal) toxicity studies provide a relatively good estimate of the potential local or systemic toxicity of a single dose to man. The results can help define the severity and range of toxicity according to dose, species, and sex and may suggest the need for subchronic tests. The results should include descriptions of the clinical signs of toxic effects at all dose levels in addition to the determination of the LD_{50}. The slope of the dose-response curves can be used to estimate safe levels of exposure for man. A steep dose curve indicates a relatively narrow range of effect doses, and therefore the estimation of the safe exposure level will be more accurate than if there is a shallow dose-response curve. The shallow dose-response curve, particularly if toxicity is delayed, suggests a complex biological action of the chemical and therefore a more variable individual response to the chemical (Casarett 1975).

The results of these tests give an indication of the clinical signs of toxicity produced by the material and may help to determine the potential risk to humans following oral, dermal, ocular, or inhalation exposure. There are limitations of these tests that need to be considered when extrapolating to human exposure conditions.

- There are many species-sex variables that make interpretation of the tests difficult.
- Variable results can be produced depending on the vehicles used in the tests.
- High doses may alter normal metabolic pathways because of overloading of metabolic enzymes and/or exhaustion of substrates required for conjugation.
- Results from single-dose studies are not reliable for predicting the hazards associated with continuous low-dose exposure.
- Results from one route of administration may be difficult to extrapolate to other routes.
- Tests should still detect significant potential hazard, although inter- and intralaboratory variation may occur.

From acute toxicity studies there are no universal triggers that can be used as guides to require subchronic studies. The determination of the need for subchronic studies should involve not only the magnitude of the dose but also the results of close observation of the animals. Delayed deaths may be the result of biochemical,

hematologic, or anatomical changes or of persistence of the chemical in the body. However, these changes may be due to the excessive doses used to produce lethal effects. If signs of toxicity persist at the end of the observation period of 7 or 14 d, particularly among animals at the lower dose levels, further investigation, including subchronic studies, may be necessary.

6.4 Subchronic Toxicity

Subchronic (90-d) toxicity studies provide a good estimate of a chemical's potential hazards. These studies reveal essentially the same toxicity pattern (with the possible exception of ocular and oncogenic effects) as a chronic studies, although the no-observable-effect dose will be greater than the chronic no-observable effect dose. This should be considered when evaluating the hazard and assessing the risk associated with the use of the chemical.

Because extensive biochemical, hematological, and anatomical studies are included in the subchronic experiment, certain reactions may trigger the need for additional studies. Also, because the dose range used in the subchronic studies includes both toxic and nontoxic doses, there inevitably will be changes in one or more of the large number of laboratory tests used as well as in the pathology studies. These changes must be evaluated carefully. Only if they occurred with increasing severity from the low-dose level to the high-dose level should additional studies be considered. Examples of such triggers are hyperglycemia, particularly with changes of the beta cells of the pancreatic islets; alternation of the differential white cell count, particularly if a neutropenia is evident; decreased testicular weight, particularly with a decrease in the number of sperm; and unusual hyperplastic change beyond that which could be expected as a physiological alteration.

Conventional subchronic studies are of limited value in defining potential adverse effects on the areas of behavior, species variation, reproductive physiology, and immunotoxicology. Furthermore, the conventional subchronic study is of virtually no value in defining potentially adverse effects in the areas of teratogenesis, genetic effects, longevity, and neoplasia.

6.5 Special Considerations

6.5.1 Reproduction and teratology

Changes in reproductive tissues as determined in subchronic studies may suggest the need for fertility and/or reproduction studies. The selection of the maximum dose for teratology studies is critical, because a maternal toxic dose may indirectly cause fetal malformations. Therefore, teratology studies should be done only after completion of subchronic studies.

6.5.2 Recovery

Recovery studies may be needed to determine if an adversely affected parameter returns to normal after termination of exposure. The lack of recovery at low doses may dictate the need for additional long-term studies usually at the same and lower dose levels.

6.5.3 Pharmacokinetic and metabolic data

One has to make a judgment about the need for pharmacokinetic and/or metabolic data. These data can be used to facilitate or clarify human risk assessment or to select the appropriate species for subchronic studies. In this instance, studies of absorption, distribution, metabolism, and elimination (ADME) on pertinent species with particular emphasis on biologic half-life may be appropriate. In specific cases, pharmacokinetic studies, including the identification of important metabolites, may be appropriate. The need for metabolic studies should be determined by sound scientific judgment based on the expected exposure of humans and the degree and extent of toxicity observed in animals.

It is clear that there are scientific and economic bases for deciding what species will be used. From a scientific reference point, one would recommend the use of that species for which the ADME is most like that of the human (if this can be deduced). The economic bases for selecting species, cost and availability, may influence the decision.

6.6 Dermal and Ocular Studies

In the industrial situation, unless adequate protection is provided, the most likely exposure to chemicals (excluding inhalation) is by dermal and/or ocular exposure. Dermal exposure is particularly important because of the potential for systemic exposure by penetration of the chemical through the skin. Although the rabbit is the most frequently used species, its skin is more permeable than human skin for most compounds (Bartek, La Budde, and Maibach 1972). The animals in which characteristics of cutaneous penetration most resemble those in the human are the rhesus monkey and the domestic swine (Wester and Maibach 1977). Other aspects of potential toxicity due to dermal exposure are irritation (Mathias and Maibach 1978), contact sensitization (Magnusson and Kligman 1977; Marzulli and Maibach 1977), phototoxicity (Maibach and Marzulli 1977), photoallergy (Harber and Shalita 1977), hypopigmentation or hyperpigmentation (Gellin, Ring, and Maibach 1977), and chloracne (National Academy of Sciences 1977). In these various tests, extrapolation to the human requires consideration of species, site of application, dose, vehicle, and method of application. Some of the limitations of dermal studies that affect extrapolations are as follows:

- Rabbit skin is more permeable than human skin for most compounds.
- Manyfold differences in permeability occur from the least to the most permeable anatomic sites.
- Nonperspiring rodent or rabbit skin does not directly mimic human skin.
- Sufficient information is often unavailable for extrapolating from the mouse, guinea pig, hamster, or dog to the human.
- Use of occlusive dressings on test animals exaggerates human exposure.
- Duration of exposure (i.e., 24 h) in test animals exaggerates human exposure (i.e., 8 h/d).

Eye irritation tests (Draize 1959) give an index of eye irritation potential in the absence of human exposure. These tests should be used for comparison with groups

of closely related compounds for which human data are available. In the rabbit test a positive result should be assessed along with other factors affecting human exposure such as conditions of use and misuse. When the test results in the rabbit suggest potential hazard, tests in the rhesus monkey may be useful as an aid to evaluate hazard for humans (Buehler and Newman 1964; Beckley, Russel, and Rubin 1969).

6.7 Conclusions

Valuable information for assessing the risk of a chemical can be obtained from acute and subchronic studies in animals. The doses used in such studies (nontoxic to toxic levels) and the duration of the subchronic study (about 90 d), with a wide variety of laboratory tests and ultimately pathology, provide a good evaluation of the toxicologic attributes of that chemical and may also determine whether additional studies are necessary. Almost invariably acute systemic toxicity studies would also be available if a subchronic study is to be done, because the need for a subchronic study is usually indicated by the results of an acute study, which is also necessary to permit a more scientific selection of dose levels for the subchronic study. Once the toxicologic attributes of a chemical are known, certain risk evaluations can be made on the basis of the type of toxicity and the possible exposure of humans. Acute toxicity studies by themselves can be of some value, particularly for single exposure for the human, but they are unreliable for more than a suggestive risk if continued exposure is probable. The dermal and ocular acute tests again are useful for very short-term exposure that most likely would occur in the use of the chemicals. However, single dermal exposure would be of little, if any, value for the determination of systemic toxicity unless penetration is so rapid that substantial blood levels are obtained with consequent potential toxic effects.

7. UNRESOLVED ISSUES AND RESEARCH RECOMMENDATIONS

The committees identified the issues and research needs associated with subchronic testing. The issues identified are the ones that could not be resolved because of a lack of literature and time. The committees were to recommend the appropriate research solutions for these areas and issues. They are as follows:

7.1 Unresolved issues

1. The use of nonrodent test species: Available literature should be reviewed to determine whether nonrodent species reveal unique or different signs of toxicity with sufficient frequency to warrant their routine use. This would include published information and data solicited by a general appeal to academic institutions, governmental agencies, and industrial concerns.
2. Duration for dog studies: It is recommended that comparisons of the literature data from dog studies in three-month and two-year durations can and should be made. If the former adequately predict the latter, the dog should be included as a species in the subchronic studies and not in nononcogenic chronic studies.
3. Number of animals: The exact number of test animals and the associated appropriate statistical treatments should be evaluated.

4. Relationship between chemical class and toxic effect: A data base needs to be generated that will aid in the correlation of specific tissue effects with chemical structure and activity. This will aid the prediction of toxic effects and assist the investigator in the protocol design for specific chemicals. Also, it would be useful to include similar information on chemical classes and organ systems.
5. Extrapolations from route to route (factors affecting the feasibility):
   a. An in-depth review of the literature should be undertaken to find data where the same chemical has been evaluated by different routes of exposures. This review should include acute, subchronic, and chronic data.
   b. A critical evaluation of such available data should be undertaken to establish for specific materials, or classes of compounds, whether target organs and overall animal response are comparable by the two routes.
   c. If variable dose and quantitative response data are available, a reasonable basis for extrapolation may be developed for individual materials and possibly groups of chemical compounds.

7.2 Research Recommendations

1. Comparative experimental studies of 14-, 30-, and 90-d durations are needed to evaluate the usefulness of the shorter period. These studies could include comparisons of various arrangements of toxicity parameters (e.g., enzyme function tests, hematology tests, pathology) that would help determine the appropriate tests necessary for the different durations.
2. Research is needed to determine the applicability of pharmacokinetic methods to the development of models for interspecies extrapolation. Differences in the responses of species to specific chemicals can be related to differences of half-lives of the parent compounds or toxic metabolites. In addition, the route of elimination can vary between species with an increase of toxicity resulting in specific organs of elimination in one species and not another. Pharmacokinetic methodology could also reveal the overloading of enzyme systems that may be deficient in one species as compared to another.
3. The similar metabolism of a compound in animal species suggests that their biological responses to toxic chemicals may be similar. Therefore, a rapid in vitro metabolic experiment using microsomal enzyme preparations from the liver or other indicated organs from several animals, including man, may aid in the selection of the most suitable animal for subchronic experiments. A data base should be compiled that lists known differences in biological response between species, including humans, and correlates these with species differences in ADME.
4. To evaluate the potential use of oral studies to determine inhalation effects, acute, two week, and subchronic 90-d studies should be performed by both routes. Included in these studies should be pharmacokinetic data to ascertain the development of steady state blood levels and tissue distribution. Histopathological data should be obtained on both short-term and subchronic studies. These data should be used to demonstrate the validity of extrapolation from one route to another and to develop extrapolation factors.
5. Research is needed to determine the degree of species specificity for the functional and biochemical tests. Because these tests were originally used for

diagnosis of human diseases, their applicability to different test species and organ function interpretation should be evaluated.

6. The value and utility of reversibility studies in a subchronic protocol design needs to be determined, utilizing data from existing literature for many chemical classes.
7. The evaluation of electroencephalograms in dogs is only a relatively recent art, and insufficient information is available to allow accurate evaluation. Future research in this area would serve to rapidly increase the store of knowledge for this potentially important toxicologic criterion.
8. Methodology for evaluation of audio toxicity should be developed.
9. Methodologies should be developed for evaluating memory and learning and their interpretation. Because of the increasing number of chemicals that could potentially affect the higher centers and because of the subtlety of such effects, techniques to detect early and minor alterations in memory and learning ability need to be developed and applied on a practical and routine basis.
10. Experimental research is needed to correlate induced sedation and aggressiveness with changes in the central nervous system. The changes in the central nervous system could be either anatomical (destruction of specific areas in the brain) or changes in the production of chemicals that affect the nerve conduction and synaptic end points. Little attention has been paid to the central nervous system effects during the administration of a chemical, and sedation or other effects have been accepted without consideration of the mechanism involved, except in the case of pathological lesions.
11. Testing and assessment procedures for cardiovascular toxicity should be developed, particularly in regard to the causative chemical classes of compounds.
12. The mechanisms of intrahepatic cholestasis and its relationship with hepatic damage should be studied. This is especially important, because this effect cannot be predicted accurately by animal studies and because it occurs frequently in humans.
13. Immunotoxicology methods need to be developed and evaluated for their potential utility. At the present time, immunology is in the stage of basic research. Such basic research does not always provide the practical methods necessary for toxicology. An adequate basic group of immunologic techniques should be developed that will highlight potential adverse effects on the immune system and thus trigger the need for further studies.
14. Chemically induced hyperplastic changes in the mammary gland, urinary bladder, and liver need to be studied to determine their relative significance. Because these organs, particularly the mammary gland and the liver, respond to various physiological stimuli, cellular activity varies within the wide normal limits. The point at which normal hyperplasia becomes abnormal is difficult to determine but is important relative to evaluating whether a specific change is approaching preneoplastic or neoplastic transformation. These hyperplastic changes need to be studied under specific highly controlled conditions in order to develop criteria that will permit a better evaluation of cellular changes as related to the detection of carcinogens.
15. The influence of chemical exposure upon spontaneous disease, such as nonspecific pneumonitis, should be determined. This type of drug effect is

important not only in the conduct of toxicology studies, but also as regards the potential activity of the chemical during exposure of man. Occasionally during subchronic and chronic toxicity studies, a chemical may appear to affect the incidence or severity of spontaneous diseases. However, the incidence and severity of spontaneous disease can vary considerably in animals so that a determination of chemical effect becomes difficult.

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APPENDIX

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S0272-101

REPORT DOCUMENTATION PAGE	1. REPORT NO. EPA 560/11-80-028	2.	3. Recipient's Accession No.
4. Title and Subtitle: Proceedings of the Workshop on Subchronic Toxicity Testing. Denver, Colorado, May 20-24, 1979.			5. Report Date: November, 1980 (date of issue)
			6.
7. Author(s): Norbert Page, Daljit Sawhney, OPTS; and Michael G. Ryon, ORNL			8. Performing Organization Rept. No. ORNL/EIS-189
9. Performing Organization Name and Address: Information Center Complex, Information Division, Oak Ridge National Laboratory, Oak Ridge, TN 37830			10. Project/Task/Work Unit No.
			11. Contract(C) or Grant(G) No. (C) IAG-80-D-X0453 (G)
12. Sponsoring Organization Name and Address: Office of Pesticides and Toxic Substances, U.S. Environmental Protection Agency, Washington, D.C. 20460			13. Type of Report & Period Covered: Final
			14.

15. Supplementary Notes

16. Abstract (Limit: 200 words)

This workshop was held at Denver, Colorado, May 20-24, 1979, to assist the Office of Pesticides and Toxic Substances in developing guidelines for subchronic toxicity testing under the Toxic Substances Control Act. The participants were organized into committees to discuss the relationship of protocol design to chemical class, the experimental design (route of exposure, dose, duration, test species, and age and number of animals), the evaluation of toxic effects (clinical observations, clinical laboratory tests, and pathology), the criteria for data extrapolation from one route of administration to another route, and limitations of acute and subchronic tests. Research recommendations submitted by each committee for their topic areas are presented in the document.

17. Document Analysis a. Descriptors

Dosage, Time, Extrapolation, Methodology, Tests, Pathology, Laboratory animals, Subchronic Toxicity, Health

b. Identifiers/Open-Ended Terms: EPA Denver Workshop, Subchronic Toxicity Workshop

c. COSATI Field/Group: 06/T

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Release Unlimited	Unclassified	65
	20. Security Class (This Page) Unclassified	22. Price

(See ANSI-Z39.18) See Instructions on Reverse OPTIONAL FORM 272 (4-77) (Formerly NTIS-35) Department of Commerce

☆ U.S. GOVERNMENT PRINTING OFFICE 1980 740-062-373